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TYPE OF REPORT: Ø|æÅaå^} å^ {

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Fort Detrick, Maryland 21702-5012

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# REPORT DOCUMENTATION PAGE

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1. REPORT DATE (DD-MM-YYYY) 01-11-2011		2. REPORT TYPE Final Addendum		3. DATES COVERED (From - To) 24 SEP 2010 - 23 Oct 2011	
4. TITLE AND SUBTITLE Treatment of Laser-Induced Retinal Injury and Visual Loss Using Sustained Release of Intra-vitreal Neurotrophic Growth Factors				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-07-1-0686	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Randy Kardon  E-Mail: randy-kardon@uiowa.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Iowa Iowa City, IA 52242				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Abstract on next page.					
15. SUBJECT TERMS Subject terms on next page.					
16. SECURITY CLASSIFICATION OF:  a. REPORT U			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 51	19a. NAME OF RESPONSIBLE PERSON USAMRMC  19b. TELEPHONE NUMBER (include area code)
b. ABSTRACT U					
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## **14. ABSTRACT**

Laser injury to the superior or inferior region of the retina results in structural and functional retina deficits. Damage to the superior retina resulted in an intrinsic up-regulation of brain-derived neurotrophic factor (BDNF), glial cell-line derived neurotrophic factor (GDNF) and a trend towards increased ciliary neurotrophic factor (CNTF) expression between three and 14 days after injury.

Intravitreal injection of growth factor-containing seemed to have some protective effect on both structural and functional properties of rods in laser-injured retinas. GDNF was somewhat more effective when compared to CNTF. However, GDNF seemed to have a negative effect on cone function (decreased photopic a-and b-waves and photopic flicker response). Even though GDNF preserved peripapillary nerve fiber layer thickness, none of the growth factors had positive effect on the function of retinal ganglion cells. On the contrary, both CNTF and GDNF seemed to have additional negative effect in addition to laser damage on RGC function. The endogenous expression of GDNF and BDNF were significantly correlated with several functional and structural retinal parameters.

## **15. SUBJECT TERMS**

Laser Injury, Neurotrophic growth factors, neuroprotection, retina function

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## Report-Year 1

### A) Methodology

**Animals:** All experimental procedures using animals were carried out in accordance with the guidelines and approved by the Iowa State University Committee on Animal Care and conform to the ARVO Statement on the Use of Animals in Ophthalmic Research. Dogs (n=30) were purchased from the A-class certified vendor (Harlan) for the use in all proposed experiments in the year 1 of this project.

**Model of laser-induced retinal damage in dogs:** Adult laboratory Beagle dogs were anesthetized with isoflurane and body temperature was maintained using a heating pad. The pupils were dilated with topical 2.5% phenylephrine hydrochloride and 1% tropicamide. Diode laser (Iris Medical, 810 nm wave length) was used to place the focal burns in the superior (tapetal – non-pigmented) or inferior (non-tapetal/pigmented) retina with a goal of inducing damage to the nerve fiber layer or outer retina.

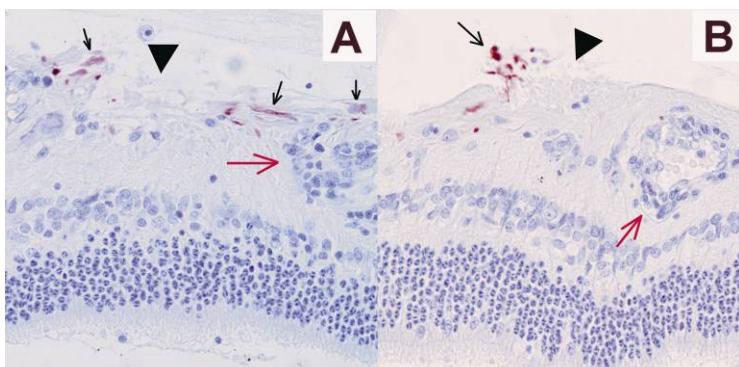
**1. The first group of animals operated in the year 1 had 30 dogs in the experiment.** This group was subdivided in 2 groups of 15 dogs. The first subgroup received the laser treatment (100 spots, 100 mW pulse energy and 200 ms pulse duration) in the inferior (non-tapetal) retina with a goal of damaging the photoreceptors and retinal pigment epithelium. The second group received the laser treatment (100 spots, 150 mW pulse energy and 200 ms pulse duration) in the superior (tapetal) retina with a goal of damaging the nerve fiber layer. **By adopting this strategy, we were able to investigate laser effect on the outer retina and nerve fiber layer in the same experimental system (canine eye).** After laser treatment, eyes will be collected at 3, 7 and 15 days postoperatively (n=5 per time point for each group) for the RT-PCR analysis and immunocytochemistry for neurotrophic factors (BDNF, CNTF, GDNF) and their respective receptors.

### B) Functional and structural characterization of the laser induced damage

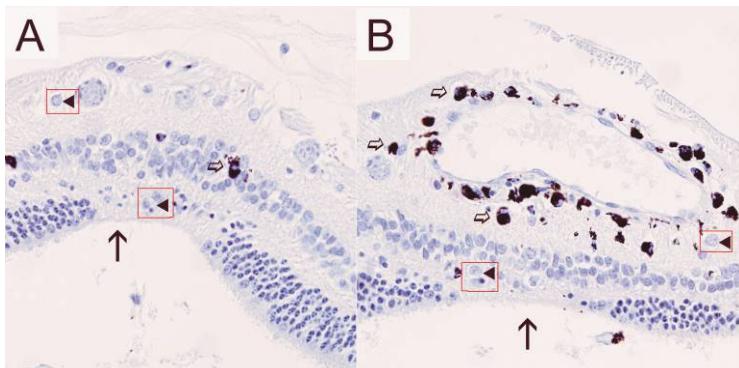
Choroidal and retinal pigment distribution in canine eyes allowed evaluation of diode laser effect (810 nm wave length) on the outer retinal layers and nerve fiber layer. Since superior retina in dogs is lacking pigment in the RPE layer, the predominant damage occurred in the inner retinal layers (particularly nerve fiber layer) mimicking effects of laser weapons with shorter wave lengths, which are characterized by more aggressive “cutting” effect on the retinal tissue (Figure 1). The inferior retina in dogs is well pigmented and predominant absorption of energy occurs in the RPE-choroid interface, resulting in damage of photoreceptors and RPE mimicking effects of laser weapons with longer wave lengths (Figure 2).

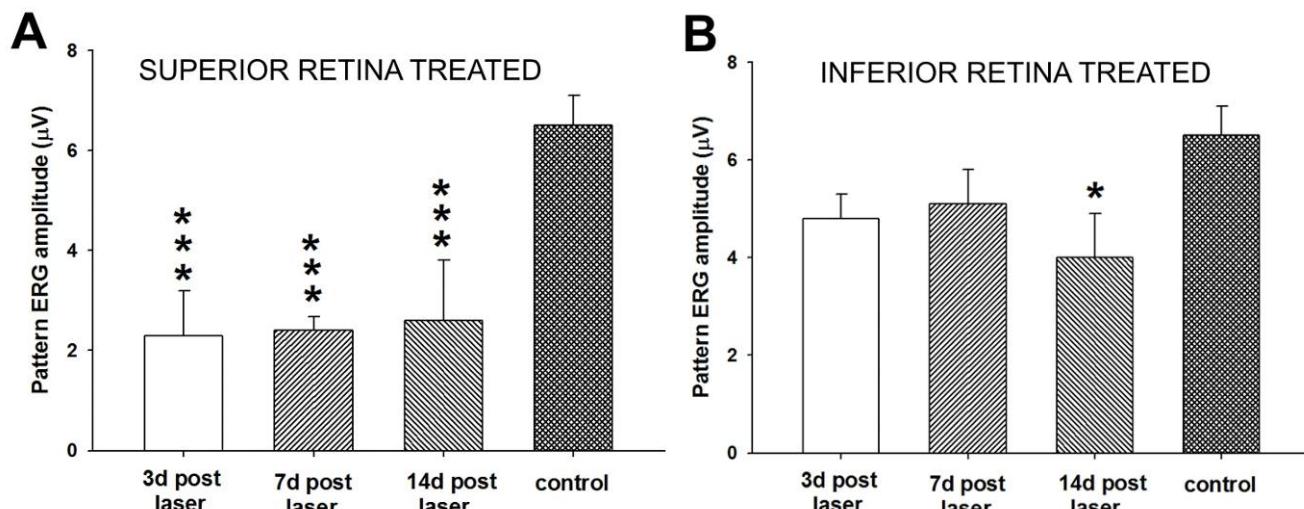
**Figure 1.** Histological changes in the nerve fiber layer of a dog with laser-induced focal damage to the inner retina. A high energy laser pulse caused focal damage to the nerve fiber layer only (black arrow heads), without causing damage to the photoreceptors in the superior (tapetal) retina. Both slides are stained for GFAP

(glial fibrillary acidic protein), which is present in reactive retinal glial cells (astrocytes and Muller cells). Intensive glial reactivity was detected around sites of NFL damage (black arrows). Significant retinal perivasculär infiltration with inflammatory cells was present around retinal blood vessels (red arrows). Image A shows more prominent destruction of the retinal ganglion cells compared to image B. Due to the lack of the pigment in the superior (tapetal) retina, the laser energy predominantly caused damage to the inner retina.

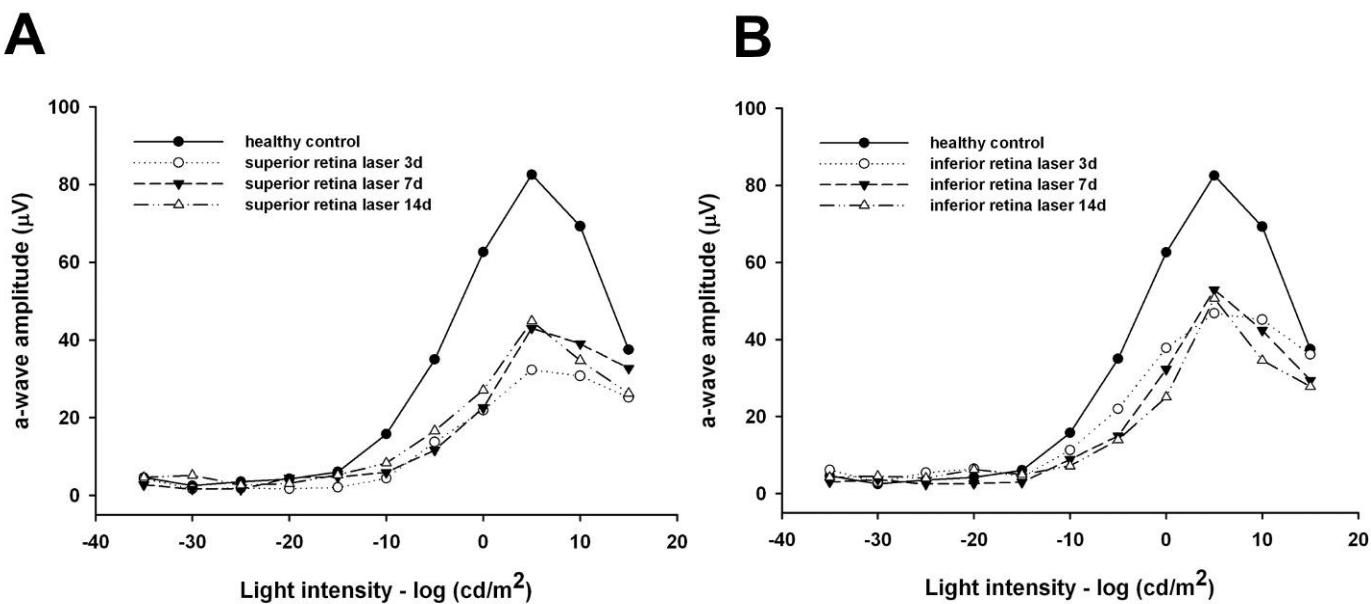


**Figure 2.** Histological changes in the outer retina of a dog with laser-induced focal damage. Medium energy-long duration laser pulse caused primary focal damage to photoreceptors (black arrows). Both slides are stained for GFAP (glial fibrillary acidic protein), which could not be detected in sites with photoreceptor damage only. Open arrows, show macrophages infiltrating retina with some phagocytosed melanin granules. Significant microglial infiltration was present in different retinal layers (arrow heads in red boxes).

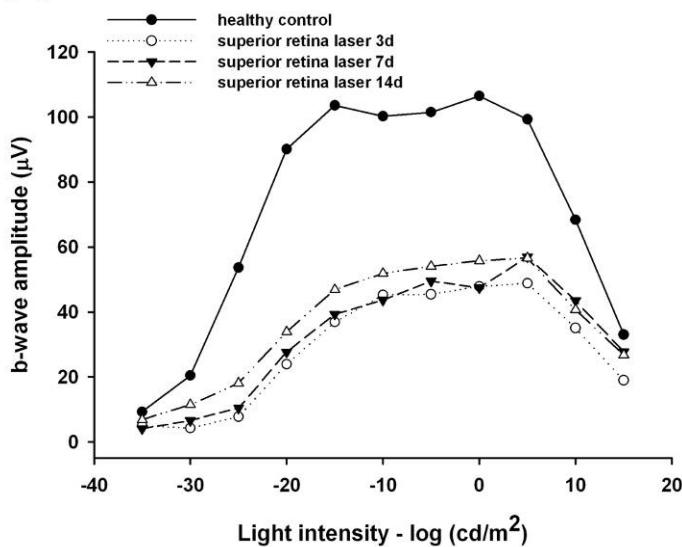
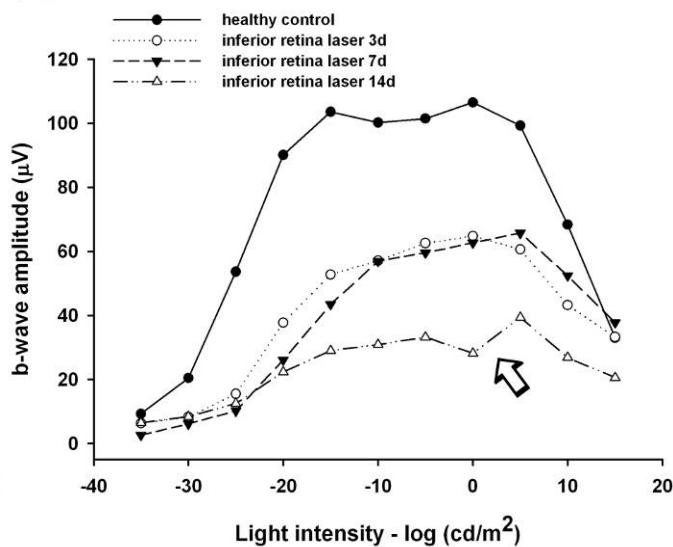




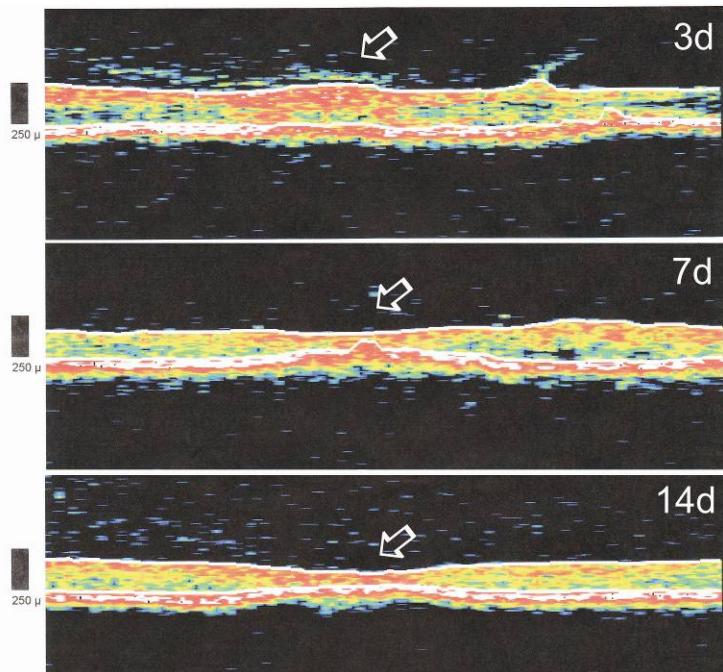
**Figure 3.** Pattern ERG (pERG) analysis showed massive deficits in the optic nerve function in dogs exposed to laser energy in the superior (non-pigmented retina), while dogs which received laser-induced damage in the inferior (pigmented) retina had some reduction in pERG amplitudes, which was not statistically significant at 3 and 7 days post laser injury, however progressed to the significant deficit at 14 days post injury, most likely as a result of progressive retinal degenerative changes. These results confirmed our hypothesis of differential site of damage in canine retinas based on pigment distribution which was also confirmed with histology and immunohistochemistry analysis (Figures 1 and 2). Furthermore we demonstrated that an immediate damage to the nerve fiber layer will result in relatively static loss of function in dogs which received laser treatment in the superior retina (no improvement or decline of function was detected up to 14 days post injury –Fig 3A).



**Figure 4.** Electrophysiology analysis of outer retina function (a-wave) in dogs which received laser injury in the superior (A) and inferior (B) retina. Despite predominant histological appearance of the nerve fiber layer damage in canine retinas which received laser damage in the superior retina, significant electrophysiological deficits were present in the outer and inner retina function, suggestive that the functional laser damage is much more widespread compared to the histology and morphology appearance of treated retinas (Figure 4A). Treatment of the inferior retina resulted in the predicted outer retina damage (deficits in the a-wave function – Figure 4B), however electrophysiology analysis showed also significant functional inner retinal function deficits (measured by decline in the b-wave function).

**A****B**

**Figure 5.** Electrophysiology analysis showed significant deficits of the inner retina function in both treated groups (b-wave amplitude analysis). While functional deficits remained static in the group which received superior retina treatment, dogs which received inferior retina damage had significant drop in function at 14 days post injury (Figure B – arrow) suggestive of the progressive degenerative changes which continued to occur beyond the initial laser damage detected at 3 and 7 days postoperatively.

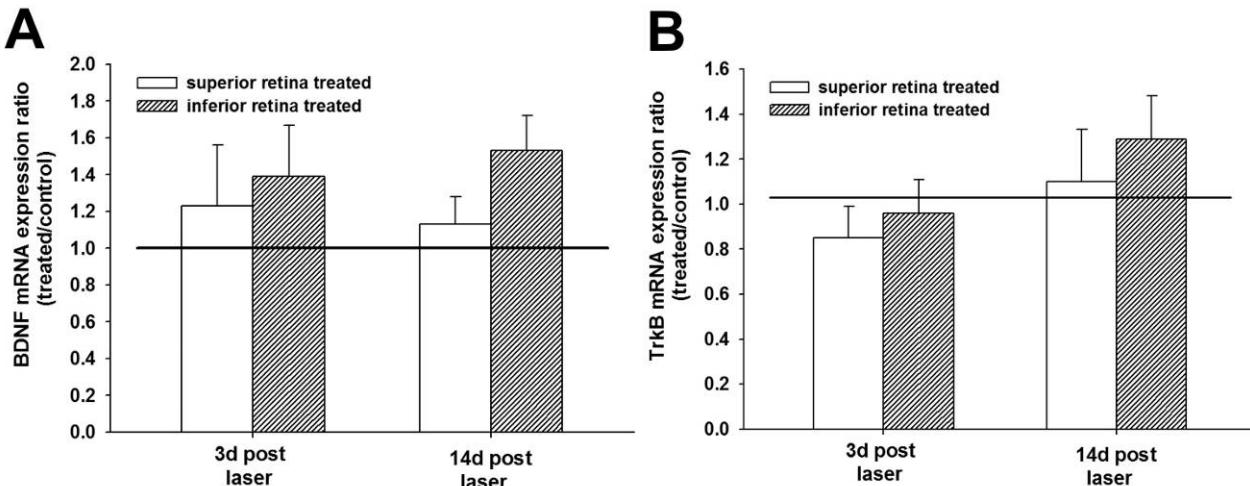


**Figure 6.** Optical coherence tomography analysis showed presence of retinal edema initially at the site of damage (3 days post injury), however retinal degenerative changes continued to progress at the 7 and 14 days post laser injury (arrow points to the site of laser damage). This set of scans is from dogs which received laser injury in the inferior (pigmented) retina. We demonstrated significant drop in the inner retina and optic nerve function 14 days post laser injury (evaluated by decrease in pERG and b-wave amplitudes – Fig 3B and 5B), and optical coherence tomography data confirmed progression of structural retinal loss which was still progressing at the last time point evaluated (14 days postoperatively).

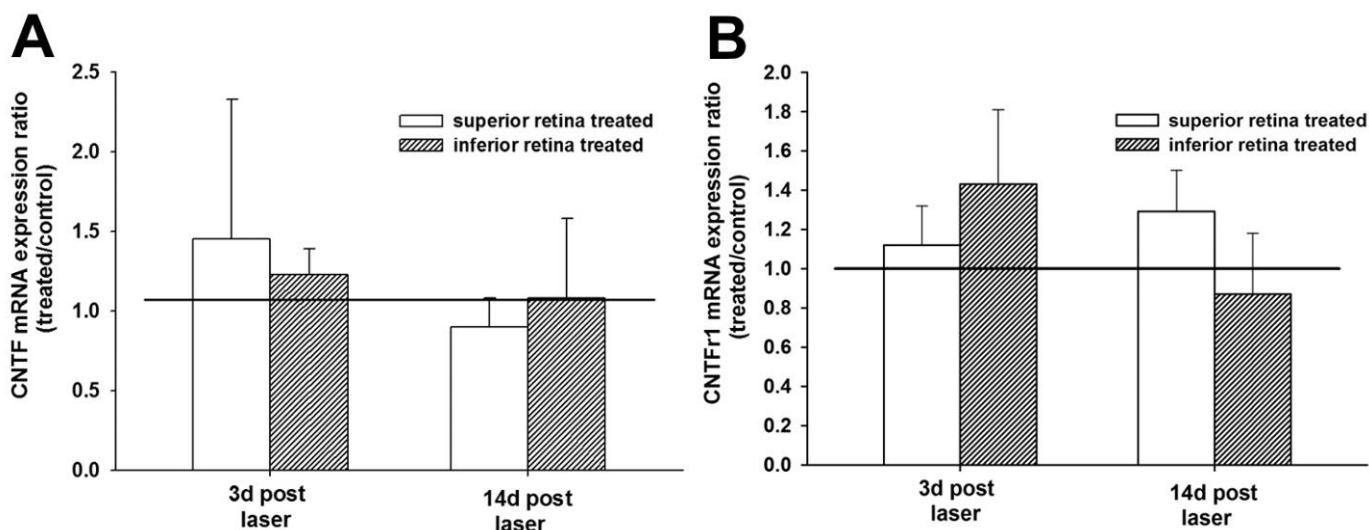
### C) Molecular characterization of growth factors and their respective receptors in laser induced retinal damage

In order to characterize expression of different neurotrophic factors (BDNF, GDNF and CNTF) and their respective receptors in the laser damaged eyes, real time PCR analysis and immunohistochemistry analysis was performed on tissue of dogs collected at 3, 7 and 15 days post laser injury. The principal purpose of these experiments was to determine the best possible growth factor candidates for treatment of laser injured eyes by following previously established rules: 1) availability of receptors for growth factors in damaged tissue; 2) absence of excessive intrinsic growth factor production, which may result in the saturation of tissue receptors and prevent positive effect of exogenously applied neurotrophic growth factors.

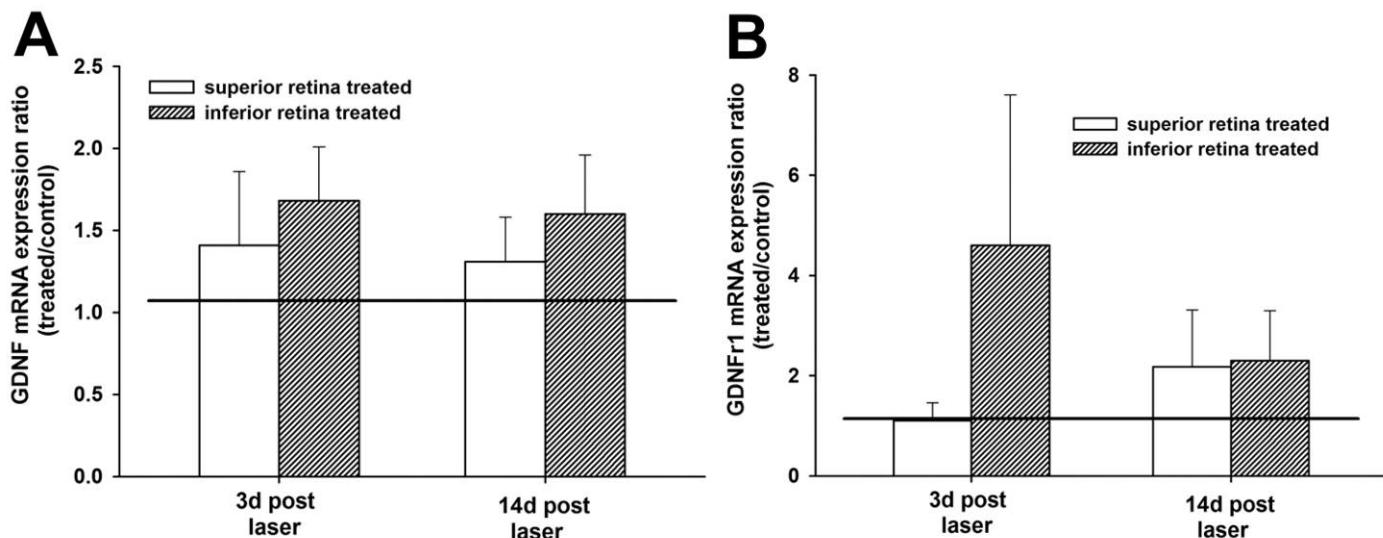
### C1) Quantitative PCR analysis of specific neurotrophic growth factors and their respective receptors



**Figure 7.** **A)** qPCR analysis showed increased expression of mRNA for brain derived neurotrophic factor (BDNF) in laser treated retinas at 3 and 14 days post laser damage. Despite predominant outer retinal damage in the inferior retina, BDNF expression was higher compared to dogs which received damage of the superior retina only. Data are calculated as a ratio between operated and control (non-treated) eyes (black line represents normalized value for control eyes – it is always positioned as a value of 1). **B)** Expression of mRNA for TrkB (respective BDNF receptor) showed decreased levels at 3 days post injury which normalized at 14 days post injury.



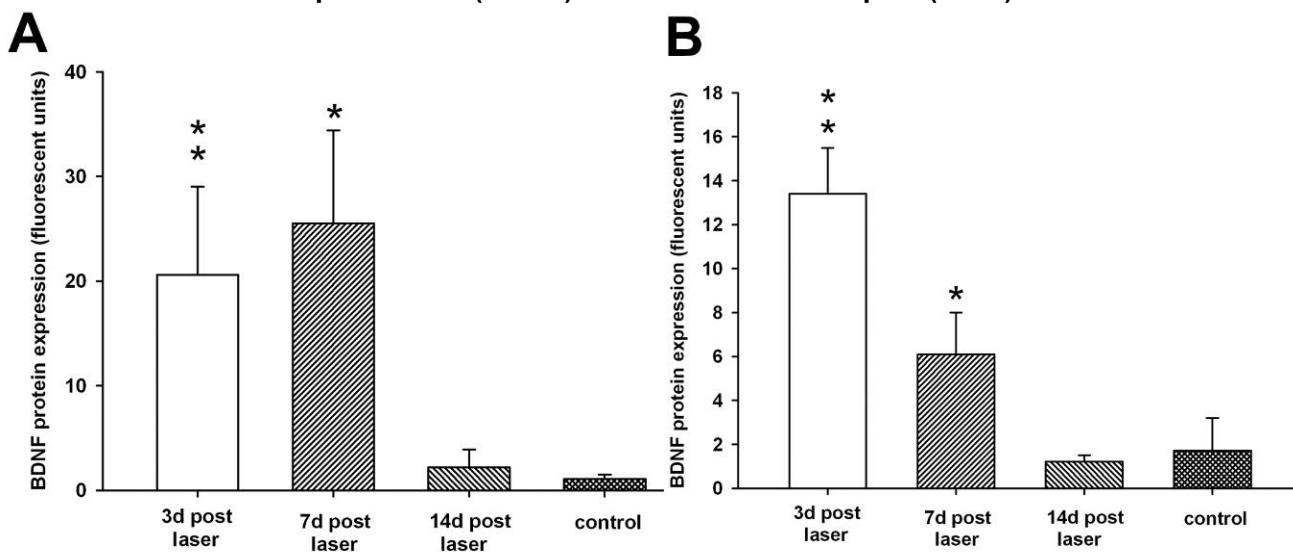
**Figure 8.** **A)** qPCR analysis showed increased expression of mRNA for ciliary neurotrophic factor (CNTF) in laser treated retinas at 3 but not at 14 days post laser damage. **B)** Expression of mRNA for CNTFr1 (respective CNTF receptor) showed mildly increased levels at 3 and 14 days post injury in dogs with superior retina damage. Dogs with inferior retina damage had higher mRNA expression levels at 3 days post injury, however expression decreased at 14 days post injury (grey bars).



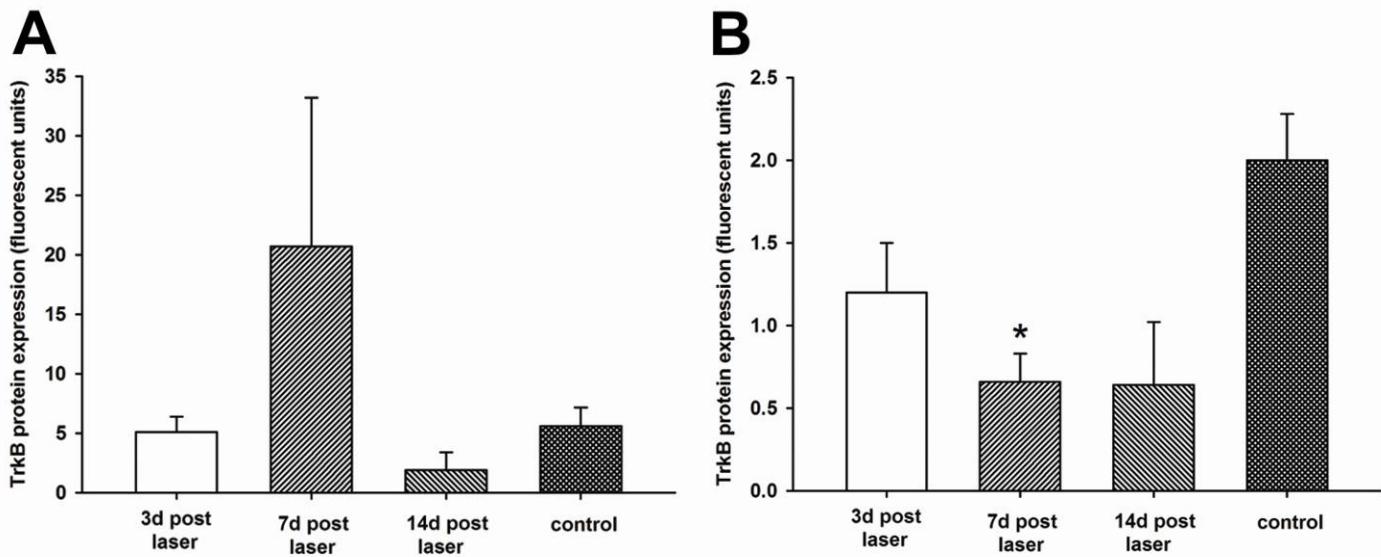
**Figure 9.** **A)** qPCR analysis showed increased expression of mRNA for glia derived neurotrophic factor (GDNF) in laser treated retinas at 3 and 14 days post laser damage. **B)** Expression of mRNA for GDNFr1 (respective GDNF receptor) showed increased levels at 3 and 14 days post injury (only exception being dogs with superior retina damage at 3 days post injury).

**C2) Semi-quantitative protein analysis of specific neurotrophic growth factors and their respective receptors**

**Brain derived neurotrophic factor (BDNF) and associated receptor (TrkB)**

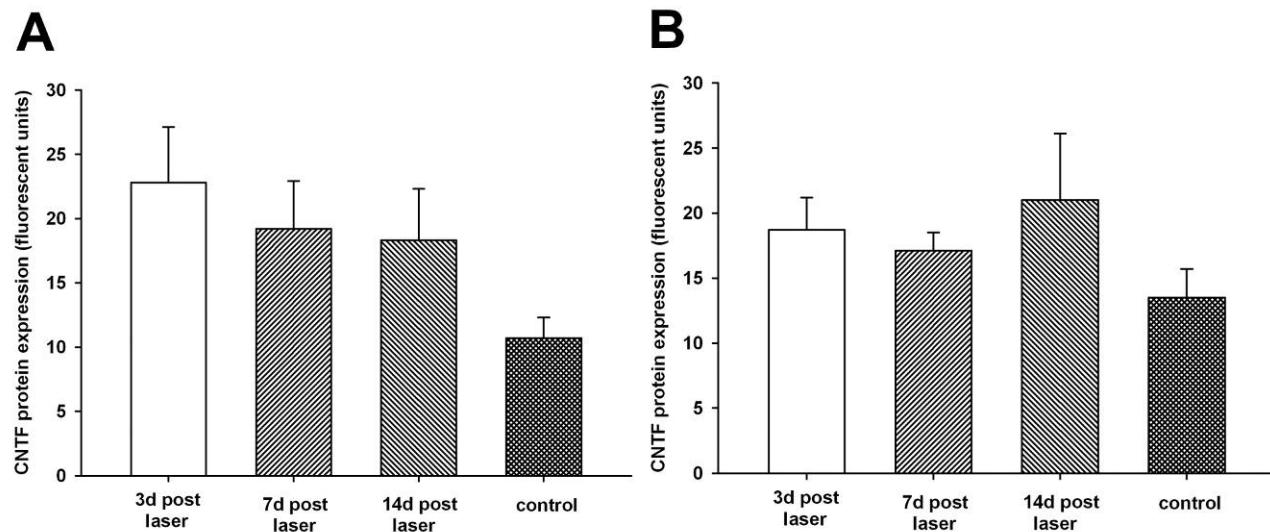


**Figure 10.** **A)** Superior retina treated - Quantification of digitalized immunohistochemistry images showed increased protein expression of BDNF in laser treated superior retinas at 3 and 7 days post laser damage, however levels normalized at 14 days post injury. **B)** Inferior retina treated – analysis showed increased protein expression of BDNF in laser treated superior retinas at 3 and 7 days post laser damage. Similar to eyes with superior retina injury, levels normalized at 14 days post injury (\* p<0.05; \*\* p<0.001, \*\*\* p<0.0001).

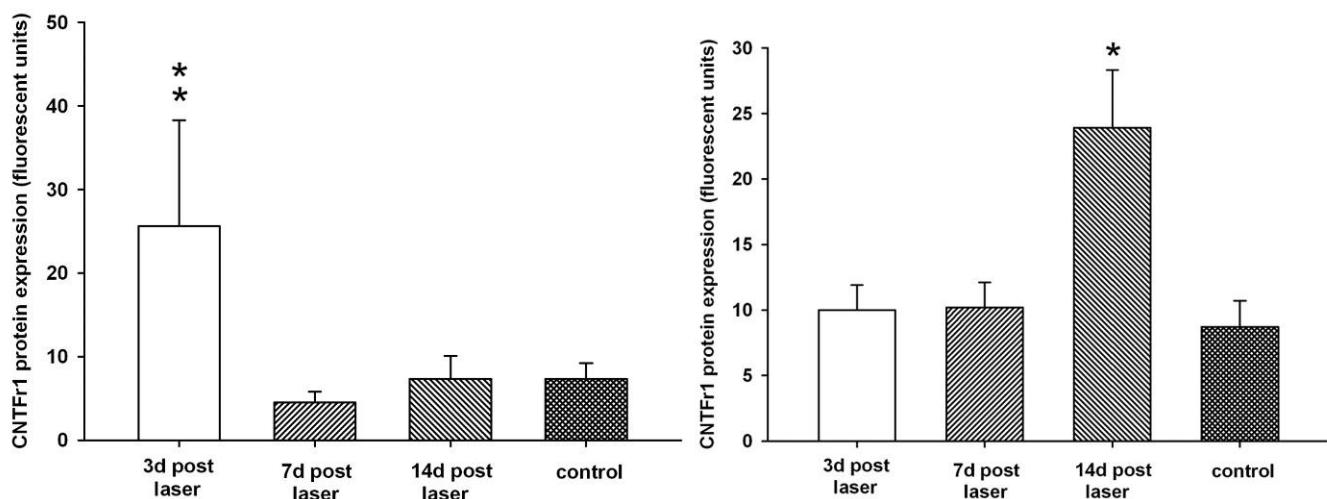
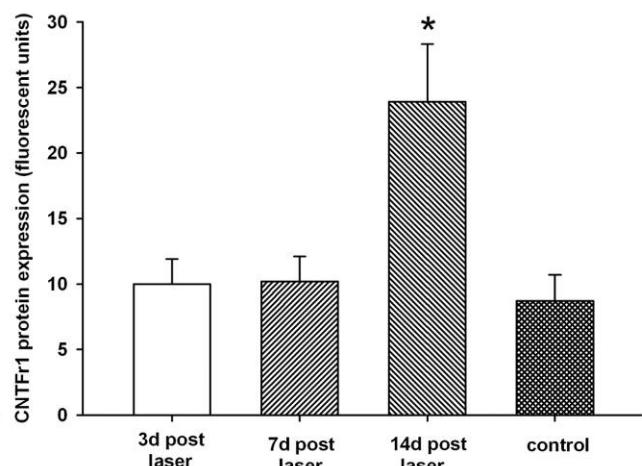


**Figure 11.** **A)** Superior retina treated - Quantification of digitalized immunohistochemistry images did not show significant change in protein expression of TrkB in laser treated superior retinas compared to control eyes. **B)** Inferior retina treated – analysis showed decreased protein expression for TrkB in laser treated inferior retinas at all time points, however difference was statistically significant only at 7 days post injury (\* p<0.05).

#### Ciliary neurotrophic factor (CNTF) and associated receptor (CNTFr1)

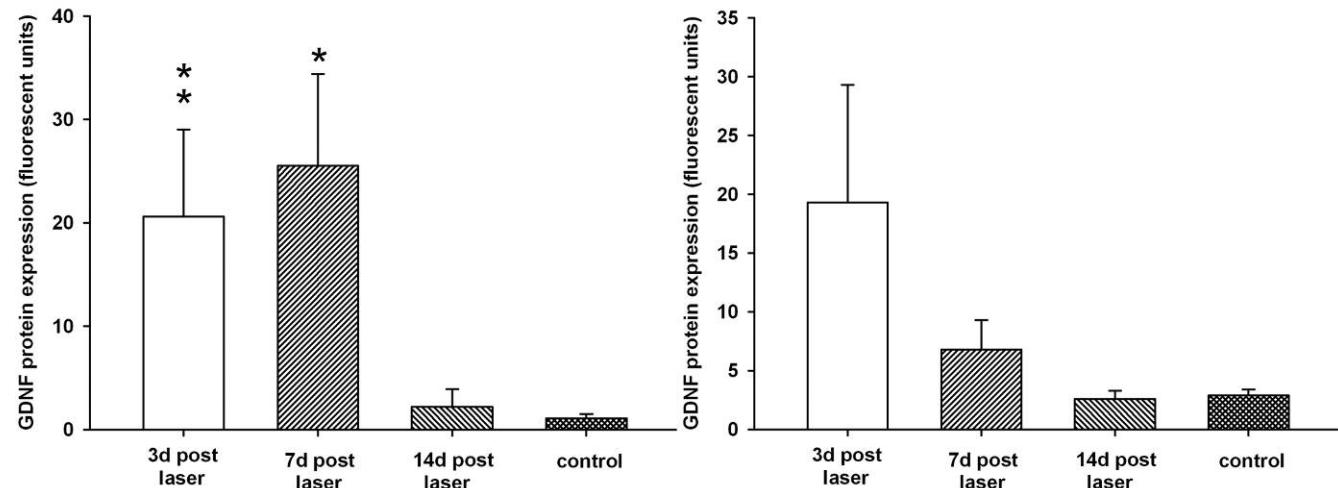
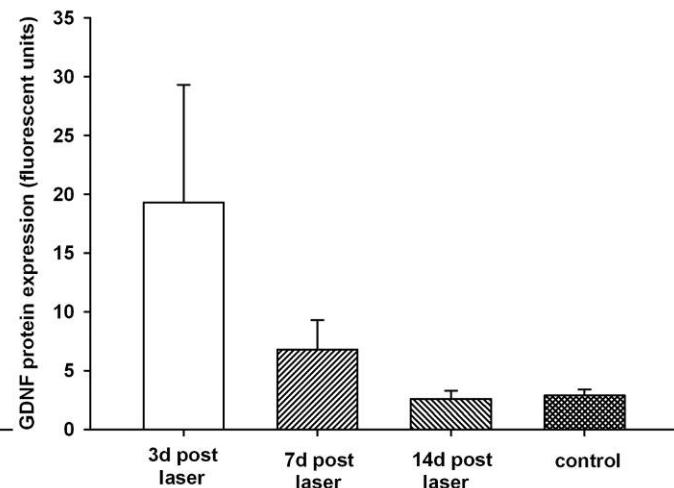


**Figure 12.** **A)** Superior retina treated - Quantification of digitalized immunohistochemistry images showed trend for increased protein expression of CNTF in laser treated superior retinas, however difference was not statistically significant when compared to control (non treated eyes). **B)** Inferior retina treated – analysis showed similar type of CNTF expression as previously observed in dogs with superior retina damage.

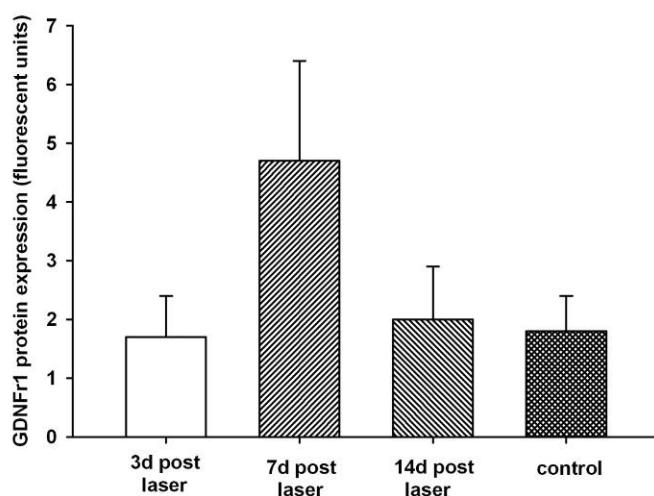
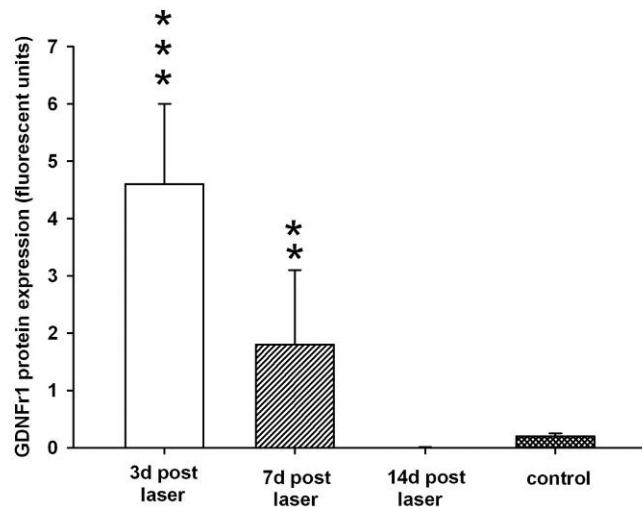
**A****B**

**Figure 13. A)** Superior retina treated - Quantification of digitalized immunohistochemistry images showed increased protein expression of CNTF receptor 1 in laser treated superior retinas at 3 days post laser damage, however levels normalized at 7 and 14 days post injury. **B)** Inferior retina treated – analysis showed increased protein expression of CNTF receptor 1 in laser treated superior retinas at 14 days post laser damage (\*  $p<0.05$ ).

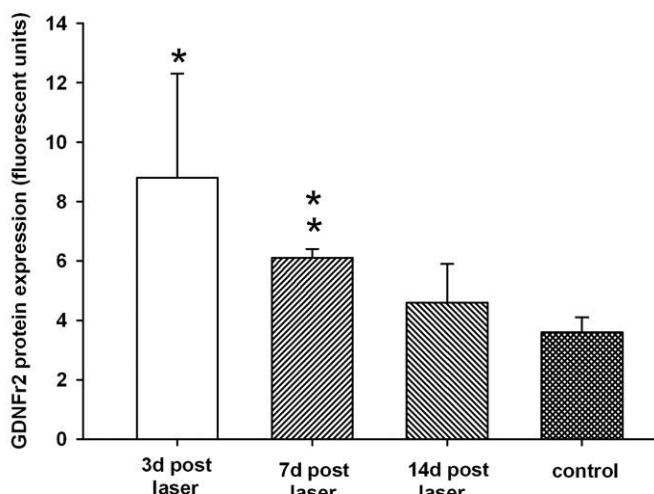
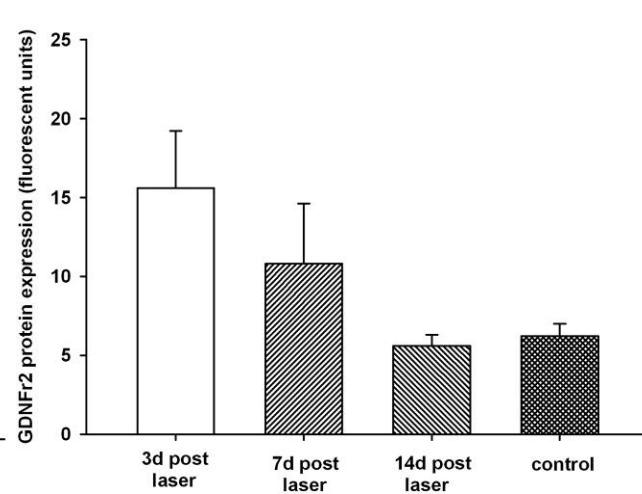
#### Glia derived neurotrophic factor (GDNF) and associated receptors (GDNFr1, GDNFr2)

**A****B**

**Figure 14. A)** Superior retina treated - Quantification of digitalized immunohistochemistry images showed increased protein expression of GDNF in laser treated superior retinas at 3 and 7 days post laser damage, however levels normalized at 14 days post injury. **B)** Inferior retina treated – analysis showed similar trend of increased protein expression (3 days post injury), however statistical analysis did not show significant difference between different treatment groups and control eyes (\*  $p<0.05$ , \*\*  $p<0.001$ ).

**A****B**

**Figure 15. A)** Superior retina treated - Quantification of digitalized immunohistochemistry images showed no change in protein expression of GDNF receptor 1 in laser treated superior retinas (trend toward increase of expression was noticed at 7 days post injury). **B)** Inferior retina treated – analysis showed increased protein expression of GDNF receptor 1 in laser treated superior retinas at 3 and 7 days post laser damage, however expression dramatically declined at 14 days post injury (\* p<0.05; \*\* p<0.001, \*\*\* p<0.0001).

**A****B**

**Figure 16. A)** Superior retina treated - Quantification of digitalized immunohistochemistry images showed increased protein expression of GDNF receptor 2 in laser treated superior retinas at 3 and 7 days post laser damage, however levels normalized at 14 days post injury. **B)** Inferior retina treated – analysis showed similar trend of increased protein expression (3 and 7 days post injury), however statistical analysis did not show significant difference between different treatment groups and control eyes (\* p<0.05, \*\* p<0.001).

GROWTH FACTOR/RECEPTOR EXPRESSION	LOCALIZATION OF THE LASER DAMAGE					
	SUPERIOR			INFERIOR		
	3d	7d	14d	3d	7d	14d
BDNF	↑↑	↑	NC	↑↑	↑	NC
TrkB	NC	T↑	NC	T↓	↓	T↓
CNTF	T↑	T↑	T↑	T↑	T↑	T↑
CNTFr1	↑	T↓	NC	NC	NC	↑
GDNF	↑↑	↑	NC	T↑	T↑	T↓
GDNFr1	NC	T↑	NC	↑↑	↑↑	T↑
GDNFr2	↑	↑	T↑	T↑	T↑	NC

**Legend:**

↑↑ - very increased expression

↑ - increased expression

T↑ - trend toward increased expression

NC - no change in expression

T↓ - trend toward decreased expression

↓ - decreased expression

**RED COLOR** – receptor expression decreased, growth factor application may not work

**BLUE COLOR** – growth factor expression increased, may saturate receptors and decrease efficacy of exogenously applied growth factor

**GREEN COLOR** – adequate expression of growth factor and/or respective receptor, exogenous supplementation should work the best

Based on results of specific growth factor (and their respective receptors) expression, we can conclude that CNTF should be excellent candidate treatment for the superior and inferior laser damage, BDNF should be candidate treatment for superior, but not inferior laser damage and GDNF should be candidate for treatment of inferior laser damage and may show efficacy in the treatment of superior laser damage.

## **SUMMARY OF THE YEAR 2 REPORT**

The primary purpose of experiments in the year 2 was to determine whether intraocular application of neurotrophic growth factors bound to biodegradable microspheres can decrease functional and structural damage to photoreceptors and retinal pigment epithelium, induced by laser damage to inferior (pigmented) canine retina. Based on results from the year 1 of this project, glia derived neurotrophic growth factor (GDNF) and ciliary neurotrophic growth factor (CNTF), were chosen as the best candidates for therapeutic treatment of laser-induced damage to photoreceptors and retinal pigment epithelium.

## Results of year 2 – summary:

### 1. Retinal structure: optical coherence tomography (OCT)

		Empty Spheres	CNTF	GDNF	Laser Only
NFL superior	14 days	↑ ***	↑ **	↑ ***	↑ ***
	30 days	↓ *	↑ **	↑ ns	↑ ***
	90 days	↑ *	↑ ***	↑ ns	↑ ***
	180 days	↑ **	↑ ns	↑ **	↑ ***
PR superior	14 days	↓ ***	↓ ns	↓ **	↓ ns
	30 days	↓ *	↓ **	↓ ns	↓ *
	90 days	↓ ***	NC	↓ *	↓ ns
	180 days	↓ ***	↓ *	↓ **	↓ ***
RT superior	14 days	NC	↑ ***	NC	↑ *
	30 days	↑ *	↑ ***	NC	↑ *
	90 days	NC	↑ ***	↑ **	↑ *
	180 days	↑ *	↑ *	↑ **	NC
NFL – C Mean	14 days	NC	↑ *	NC	NC
	30 days	NC	NC	NC	NC
	90 days	NC	NC	NC	NC
	180 days	↓ ns	↓ ***	↑ ns	↓ ***
NFL – C temporal	14 days	NC	↑ ns	NC	↑ ns
	30 days	NC	NC	↓ ns	↑ ns
	90 days	NC	NC	NC	NC
	180 days	NC	↓ ***	↑ ns	↓ ***
NFL - C superior	14 days	NC	↑ **	NC	NC
	30 days	NC	NC	NC	↑ ns
	90 days	↑ *	↑ *	NC	NC
	180 days	NC	↓ **	↑ ns	↓ ***
NFL - C nasal	14 days	NC	↑ ns	NC	↑ ns
	30 days	NC	NC	↓ ns	↑ ns
	90 days	NC	NC	NC	NC
	180 days	NC	↓ **	↑ ns	↓ ***
NFL - C inferior	14 days	↓ **	↓ ns	↑ ns	↓ ns
	30 days	↓ *	↓ *	↑ ns	↓ *
	90 days	↓ *	↓ *	↑ ns	↓ *
	180 days	↓ ns	↓ *	↑ ns	↓ **

**Table 1.** Morphological analysis (OCT) of superior retina parameters (macular (*area centralis*, superior retina) region and peripapillary nerve fiber layer (NFL) thickness) showed significant changes in retinal structure, despite laser induced retinal damage to the inferior retina only. Predominant changes included increase in retinal thickness (RT) and nerve fiber layer thickness (most likely due to the inflammation-induced edema and/or glial proliferation) in the *area centralis* (linear scans in superior retina). We also observed decreased thickness of photoreceptor (PR) layer (suggestive of possible loss of photoreceptors) in the *area centralis* (linear scans in superior retina) and decreased peripapillary nerve fiber layer thickness (circular scans around optic nerve head), suggestive of loss of retinal ganglion cell axons. GDNF appeared to have protective effect on retinal ganglion cell axons, but did not prevent loss in photoreceptor structure.

2. Retinal function: electroretinography (ISCEV ERG):

		Empty Spheres	CNTF	GDNF	Laser Only
Photopic a-wave	14 days	NC	NC	↑ ns	↓ ns
	30 days	NC	↑ ns	↓ ns	↓ *
	90 days	NC	NC	NC	NC
	180 days	NC	NC	NC	NC
Photopic b-wave	14 days	↓ *	↓ ns	↓ *	↓ ns
	30 days	↓ ns	↓ ns	↓ ns	↓ *
	90 days	NC	NC	↓ ns	↓ ns
	180 days	NC	NC	↓ ns	↓ *
Flicker response	14 days	↓ ns	NC	NC	NC
	30 days	↓ ns	NC	↓ ns	↓ *
	90 days	NC	↓ ns	NC	NC
	180 days	NC	NC	NC	↓ ns
Scotopic a-wave	14 days	↓ *	NC	↑ ns	↓ ns
	30 days	↓ **	↑ ns	NC	↓ **
	90 days	↓ **	↓ **	NC	↓ **
	180 days	↓ ***	↓ ***	↓ ***	↓ ***
Scotopic b-wave	14 days	↓ ns	NC	↑ ns	↓ ns
	30 days	↓ **	↑ ns	NC	↓ **
	90 days	↓ *	↓ **	↓ ns	↓ *
	180 days	↓ **	↓ ***	↓ **	↓ ***
Oscillatory Potentials	14 days	↓ *	↓ ns	↑ ns	↓ ns
	30 days	↓ **	↑ ns	↓ ns	↓ *
	90 days	↓ **	↓ **	↓ ns	↓ ***
	180 days	↓ ***	↓ ***	↓ ***	↓ ***

**Table 2.** Functional retinal analysis (ISCEV ERG) - all groups at different time points after laser damage to the inferior retina. We have observed decreased scotopic a- and b- waves, and oscillatory potentials. This is suggestive of the primary damage to rods (cone function was relatively normal or completely normal). Exogenously applied GDNF and CNTF seemed to have protective effect on functional properties of affected retinas (GDNF to greater extent), as long as they were present in the eye (60-90 days). Six months after laser exposure, the functional deficits were also present in CNTF/GDNF treated eyes, which is suggestive of progressive loss of retinal function that is still occurring even months after the initial laser insult.

3. Retinal ganglion cell function: Pattern electroretinography (pERG):

		Empty Spheres	CNTF	GDNF	Laser Only
pERG amplitude	14 days	↓ ns	↓ ns	↓ ns	↓ *
	30 days	↓ *	↓ **	↓ ns	↓ **
	90 days	↓ *	↓ **	↓ **	↓ *
	180 days	↓ **	↓ ***	↓ ns	↓ ns

**Table 3.** Summary for pattern ERG analysis results - all groups at different time points after laser damage. Evaluation of retinal ganglion cell function revealed decrease in all treated groups, however GDNF treated

eyes had the least amount of deficits, when compared to eyes which received laser damage and were not treated ("laser only") or were treated with empty microspheres or CNTF microspheres.

Legend for tables 1-3:

NC – no change in parameter

↑<sub>ns</sub> – increase in parameter, not statistically significant

↓<sub>ns</sub> – decrease in parameter, not statistically significant

↑ \*, ↑ \*\*, ↑ \*\*\* – increase in parameter, statistical significance \*, \*\*, \*\*\*

↓ \*, ↓ \*\*, ↓ \*\*\* – decrease in parameter, statistical significance \*, \*\*, \*\*\*

NFL – nerve fiber layer – linear scan

NFL – C – peripapillary nerve fiber layer (circular scan around optic nerve head)

PR – photoreceptor thickness

RT – retinal thickness

Conclusions of experiments for the year 2:

1. **Laser energy applied to inferior (pigmented) retina causes chronic and progressive structural deficits (decrease of photoreceptor layer thickness) in the superior (non-pigmented) retina as well.**
2. **Laser damage to inferior retina causes chronic and progressive functional deficits (decreased scotopic a- and b- waves and oscillatory potentials) in different retinal neuronal populations. These deficits predominantly affect rods (cone function was relatively non-affected).**
3. **Laser retinal damage can cause significant loss of the peripapillary nerve fiber layer thickness and can induce retinal ganglion cell deficits observed by pattern electroretinography.**
4. **Intravitreal injection of growth factor-containing microspheres (as long as they were present in the eye) seemed to have protective effect on both structural and functional properties in laser-injured retinas. GDNF was more effective when compared to the CNTF.**
5. **At conclusion of year 2 of the study (6 months after laser exposure), the functional deficits were present also in growth factor- treated eyes, which is suggestive of progressive loss of retinal function, that is still occurring even months after initial damage. These results dictate the need for repetitive intraocular injections of growth factors, or development of longer-lasting growth factor delivery systems that could be inserted in the eye to protect retina from progressive damage over the long period of time.**

## Summary of the year 3 studies

### Results – summary

		Empty	CNTF	GDNF	Laser Only
NFL inferior	14 days	↑ **	↑ ns	↑ ns	NC
	30 days	NC	NC	↓ ns	NC
	90 days	NC	↓ ns	NC	NC
	180 days	↓ ns	NC	↓ ns	↑ ns
PR inferior	14 days	NC	NC	↓ *	↓ *
	30 days	↓ ns	↓ ns	↓ ns	↓ *
	90 days	↓ ns	↓ ns	↓ ns	↓ *
	180 days	↓ *	↓ ns	↓ ns	↓ *
RT inferior	14 days	↑ ***	↑ ***	↑ **	↓ ns
	30 days	↑ ns	↑ ns	NC	↓ ns
	90 days	↑ ns	NC	NC	↓ ns
	180 days	NC	NC	NC	NC
NFL - C mean	14 days	↑ ns	↑ ns	↑ ns	↑ ns
	30 days	↑ ns	↓ ns	↑ ns	↑ ns
	90 days	↑ ns	↓ ns	↑ ns	NC
	180 days	↑ ns	↓ ***	NC	↓ ***
NFL - C temporal	14 days	↑ *	↑ ns	↑ ns	↑ *
	30 days	↑ ns	↓ ns	NC	↑ ns
	90 days	↑ *	↓ ns	NC	↓ ns
	180 days	↑ ns	↓ ***	↓ ns	↓ ***
NFL - C superior	14 days	↑ ns	↑ *	NC	↑ ns
	30 days	NC	↓ ns	NC	↑ *
	90 days	↑ *	↓ ns	NC	NC
	180 days	↑ ns	↓ ***	↓ *	↓ ***
NFL - C nasal	14 days	NC	↑ *	↑ ns	↑ ns
	30 days	NC	NC	↑ ns	↑ ns
	90 days	NC	↑ ns	↑ ns	NC
	180 days	↑ ns	↓ **	NC	↓ **
NFL - C inferior	14 days	NC	↓ ns	↑ ns	↓ *
	30 days	↑ ns	↓ ns	↑ ns	↓ *
	90 days	NC	↓ *	↑ ns	↓ *
	180 days	NC	↓ ns	NC	↓ **

**Table 1.** Morphological analysis (OCT) of inferior retina and peripapillary nerve fiber layer thickness showed significant changes in retinal structure, despite laser induced retinal damage to the superior retina only. Predominant changes induced by laser injury included decrease in photoreceptor thickness and peripapillary nerve fiber layer thickness. Shortly after laser injury and intravitreal injection, total retinal thickness and nerve fiber layer thickness in inferior retina were increased in all injected eyes (most likely due to inflammatory edema resulting from cumulative effect of laser injury and intravitreal injection). CNTF and GDNF appeared to have protective effect on photoreceptor structure. GDNF appeared to have protective effect on retinal ganglion cell axons.

		Empty	CNTF	GDNF	Laser Only
Photopic a-wave	14 days	↓ ns	↓ ns	↓ *	↓ *
	30 days	↓ *	↓ *	↑ ns	↓ ns
	90 days	↓ *	↓ *	↓ **	↓ ns
	180 days	↓ *	↓ *	↓ *	↓ *
Photopic b-wave	14 days	↓ ns	↓ ns	↓ **	↓ *
	30 days	↓ ns	↓ *	↑ ns	↓ ns
	90 days	↓ ns	↓ ns	↓ **	↓ ns
	180 days	↓ ns	↓ ns	↓ **	↓ *
Flicker response	14 days	↓ *	↓ *	↓ ***	↓ ns
	30 days	↓ *	↓ *	↓ **	↓ *
	90 days	↓ ns	↓ *	↓ **	↓ ns
	180 days	↓ ns	↓ ns	↓ **	↓ ns
Scotopic a-wave	14 days	↓ *	NC	↓ ns	NC
	30 days	↓ ***	NC	NC	↓ **
	90 days	↓ **	↓ *	↓ *	↓ ***
	180 days	↓ ***	↓ ***	↓ ***	↓ ***
Scotopic b-wave	14 days	↓ ns	NC	↓ ns	↓ ns
	30 days	↓ **	↑ ns	NC	↓ ***
	90 days	↓ **	↓ **	↓ **	↓ **
	180 days	↓ **	↓ **	↓ ***	↓ ***
Oscillatory Potentials	14 days	↓ *	↓ ns	↓ ns	NC
	30 days	↓ *	↑ ns	NC	↓ *
	90 days	↓ **	↓ **	↓ *	↓ ***
	180 days	↓ ***	↓ ***	↓ ***	↓ ***

**Table 2.** Functional retinal analysis (Full-field ERG) - all groups at different time points after laser damage. Predominant changes were decreased scotopic a- and b- waves, and oscillatory potentials. This is suggestive of the primary damage to rods (cone function was relatively normal or completely normal). Exogenously applied GDNF and CNTF seemed to have some protective effect on functional properties of affected rods (GDNF to somewhat greater extent), as long as they were present in the eye. By three months after laser exposure, when the growth factors were no longer present in the eye, the functional deficits started to show also in CNTF/GDNF treated eyes. By six months after laser exposure, the functional deficits in all eyes were comparable, which is suggestive of progressive loss of retinal function even months after the initial laser insult.

		Empty	CNTF	GDNF	Laser Only
pERG amplitude	14 days	↓ ***	↓ ***	↓ ***	↓ ***
	30 days	↓ ***	↓ ***	↓ ***	↓ ***
	90 days	↓ ***	↓ ***	↓ ***	↓ ***
	180 days	↓ ***	↓ ***	↓ ***	↓ **

**Table 3.** Summary for pattern ERG analysis results - all groups at different time points after laser damage. No treatment showed protective effect on retinal ganglion cell function at any recording time point.

Legend:

NC – no change in parameter

↑ ns – increase in parameter, not statistically significant

↓ ns – decrease in parameter, not statistically significant

↑ \*, ↑ \*\*, ↑ \*\*\* – increase in parameter, statistical significance \*, \*\*, \*\*\*

↓ \*, ↓ \*\*, ↓ \*\*\* – decrease in parameter, statistical significance \*, \*\*, \*\*\*

NFL – nerve fiber layer – linear scan

NFL – C – peripapillary nerve fiber layer (circular scan around optic nerve head)

PR – photoreceptor thickness

RT – retinal thickness

There are several conclusions which can be drawn from results of our study:

1. Laser energy applied to superior (non-pigmented/tapetal) retina causes chronic and progressive structural (decrease of photoreceptor layer thickness) deficits in the inferior retina as well.
2. Laser damage to superior retina causes chronic and progressive functional deficits (decreased scotopic a- and b-waves and oscillatory potentials and to a lesser degree deficits in photopic a- and b-waves) in different retinal neuronal populations. These deficits predominantly affect rods (cone function was affected to much less degree).
3. Laser retinal damage causes significant loss of the peripapillary nerve fiber layer thickness (loss of ganglion cell axons) and induces retinal ganglion cell deficits observed by pattern electroretinography.
4. Intravitreal injection of growth factor-containing microspheres (as long as they were present in the eye) seemed to have some protective effect on both structural and functional properties of rods in laser-injured retinas. GDNF was somewhat more effective when compared to CNTF. However, GDNF seemed to have negative effect on cone function (decreased photopic a- and b-waves and photopic flicker response). Even though GDNF preserved peripapillary nerve fiber layer thickness, none of the growth factors had positive effect on the function of retinal ganglion cells. On the contrary, both CNTF and GDNF seemed to have additional negative effect to laser damage on RGC function.
5. At conclusion of our study (6 months after laser exposure), the functional deficits were of same magnitude in all eyes, which is suggestive of progressive loss of retinal function, that is still occurring even months after initial damage. These results dictate the need for development of long-term treatment protocols to protect retina from progressive damage over long period of time.

## YEAR 3 - REPORT

The primary purpose of experiments in the year 3 of this project was to determine whether intraocular application of neurotrophic growth factors bound to biodegradable microspheres can decrease laser-induced functional and structural damage to the superior retina. In canine eyes superior retina does not have pigmented retinal pigment epithelium (RPE), so majority of damage is concentrated on the inferior retina (particularly nerve fiber layer), due to the lack of laser energy absorption by RPE. Based on results from the year 1 of this project, we have determined that ciliary neurotrophic growth factor (CNTF) should be the best candidate for treatment. Molecular profiling of laser damaged retinal tissue, also revealed that glia derived neurotrophic growth factor (GDNF) could also be potentially effective in the therapeutic treatment of laser-induced inner retinal damage.

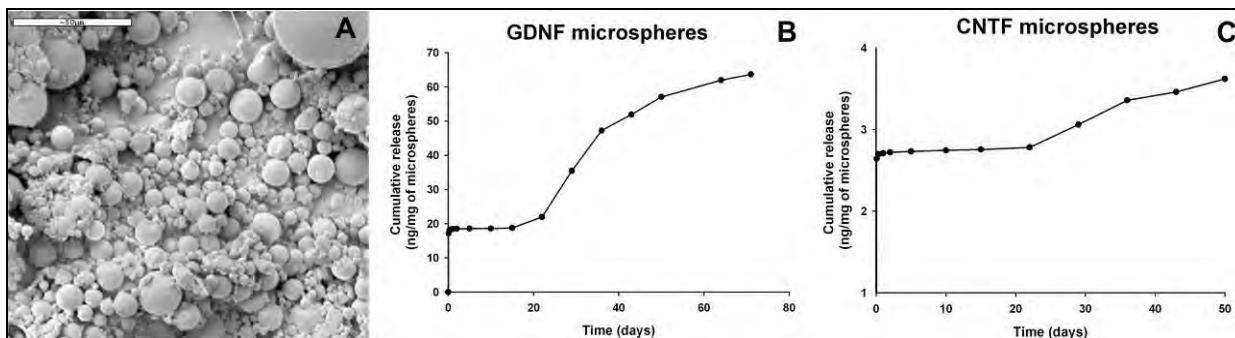
### A) Methodology

**Animals:** All experimental procedures using animals were carried out in accordance with the guidelines and approved by the Iowa State University Committee on Animal Care and conform to the ARVO Statement on the Use of Animals in Ophthalmic Research. Dogs (n=27) were purchased from the A-class certified vendor (Harlan) for the use in all proposed experiments in the year 3 of this project.

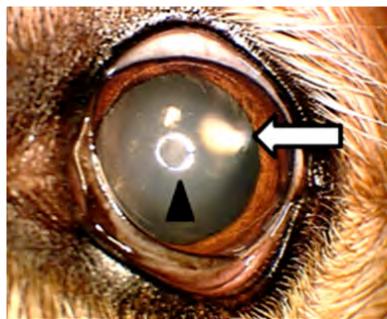
**Model of laser-induced retinal damage in dogs:** Adult laboratory Beagle dogs were anesthetized with isoflurane and body temperature was maintained using heating pad and warming blanket. The pupils were dilated with topical 1% tropicamide. Diode laser (Iris Medical, 810 nm wavelength) was used to create focal burns in the superior (tapetal/non-pigmented) retina with a goal of inducing damage to the nerve fiber layer.

**In the third year of the project, 27 dogs were included in the experiment.** Animals were divided into 4 groups of 7 dogs (6 dogs in group 4). All animals received laser treatment (100 spots positioned in the superior retina, 350 mW pulse energy and 200 ms pulse duration) in the superior (non-pigmented) retina with a goal of primarily damaging retinal nerve fiber layer (RNFL).

After laser treatment, animals in group 1 received intravitreal injection of glial-derived neurotrophic factor (GDNF) - containing PLGA polymer microspheres (Figures 1,2), animals in group 2 received intravitreal injection of ciliary neurotrophic growth factor (CNTF) - containing PLGA polymer microspheres, animals in group 3 received intravitreal injection of empty PLGA microspheres, and animals in group 4 did not receive any additional treatment (control group – laser damage only). Regular ophthalmic examinations, monitoring of general health of the animals, structural (optical coherence tomography – OCT) and functional (pattern ERG – PERG and full-field ERG) recordings of treated eyes were performed at 14, 30, 90 and 180 days after laser treatment. After the last recordings (180 days post laser treatment), animals were euthanized and eyes were collected for histopathology analysis.



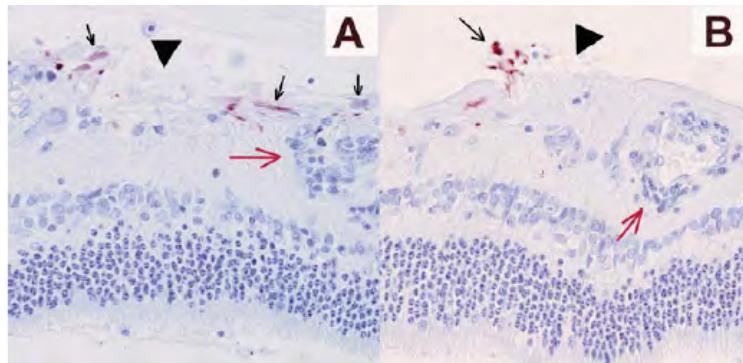
**Figure 1. Kinetics data of PLGA microspheres containing neurotrophic growth factors.**  
Pharmacokinetics of the GDNF and CNTF microspheres is shown in pictures B and C, respectively.



**Figure 2.** Clinical photograph of a dog after intravitreal injection of GDNF containing microspheres. White arrow points at white deposit of microspheres suspended in vitreous. Arrowhead points at camera flash reflection on the cornea.

### **B) Structural characterization of the laser induced damage**

The effect of diode laser on tapetal (superior, non-pigmented) retina was evaluated in year one of the experiment. The superior retina of dogs has less pigment, causing primary laser damage to the inner retina, and particularly retinal nerve fiber layer (Figure 3).



**Figure 3.** Histological changes in the nerve fiber layer of a dog with laser-induced focal damage to the superior (tapetal) retina. A **laser** pulse caused focal damage to the nerve fiber layer (black arrow heads). Both slides are stained for GFAP (glial fibrillary acidic protein, red stain on the image), which is present in reactive retinal glial cells (astrocytes and Muller cells). Intensive glial reactivity was detected around sites of NFL damage (black arrows). Significant retinal perivascular infiltration with inflammatory cells was

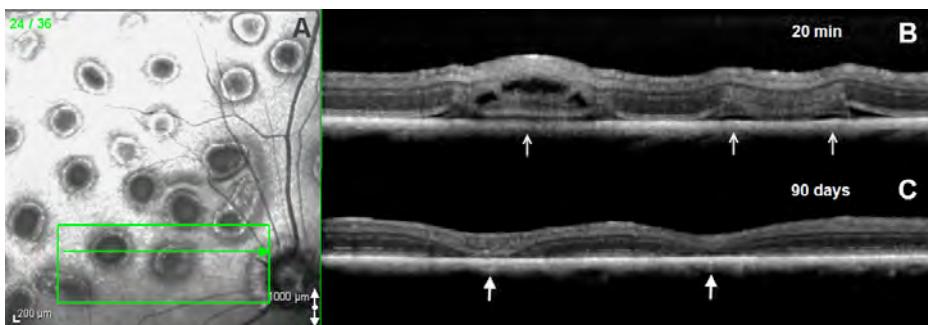
present around retinal blood vessels (red arrows). Image A shows more prominent destruction of the nerve fiber layer compared to the image B. Due to the lack of the pigment in the superior (tapetal) retina, the laser energy predominantly caused damage to the inner retina.

### **C) Results:**

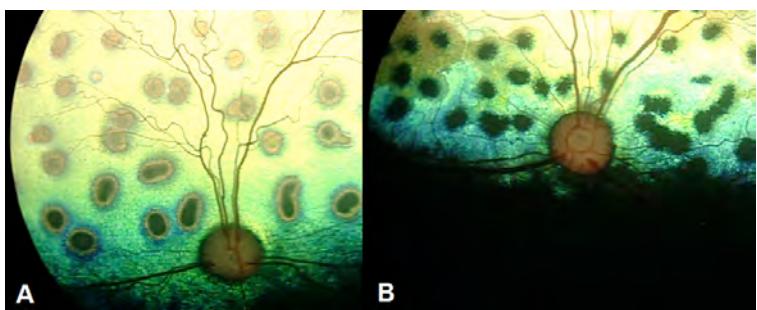
#### **1. Structural characterization of the laser induced damage**

##### **Optical coherence tomography**

Optical coherence tomography scans were performed shortly after laser treatment to document immediate retinal damage, and then at 14, 30, 90 and 180 days post laser exposure (Figure 4). Figure 5 shows retinal photographs at times corresponding to OCT scans shown in Figure 4 (immediately after laser damage and 90 days after laser treatment).



surrounding zone of exudative retinal detachment (arrows). Bottom image represents examination 90 days after laser exposure: central zones are converted in regions of retinal scars, clinically seen as pigment proliferation (arrows).



**Figure 5.** Fundus image of canine retina immediately (A) and 90 days (B) after laser exposure. A: retinal laser damage (black center) surrounded by focal retinal edema/detachment (yellow-grey halo surrounding the black center). B: lesions after 90 days – the black center represents pigment proliferation/scar formation, golden halo of hyperreflectivity around lesions represents areas of retinal degeneration.

OCT images were obtained in each recording session in following order:

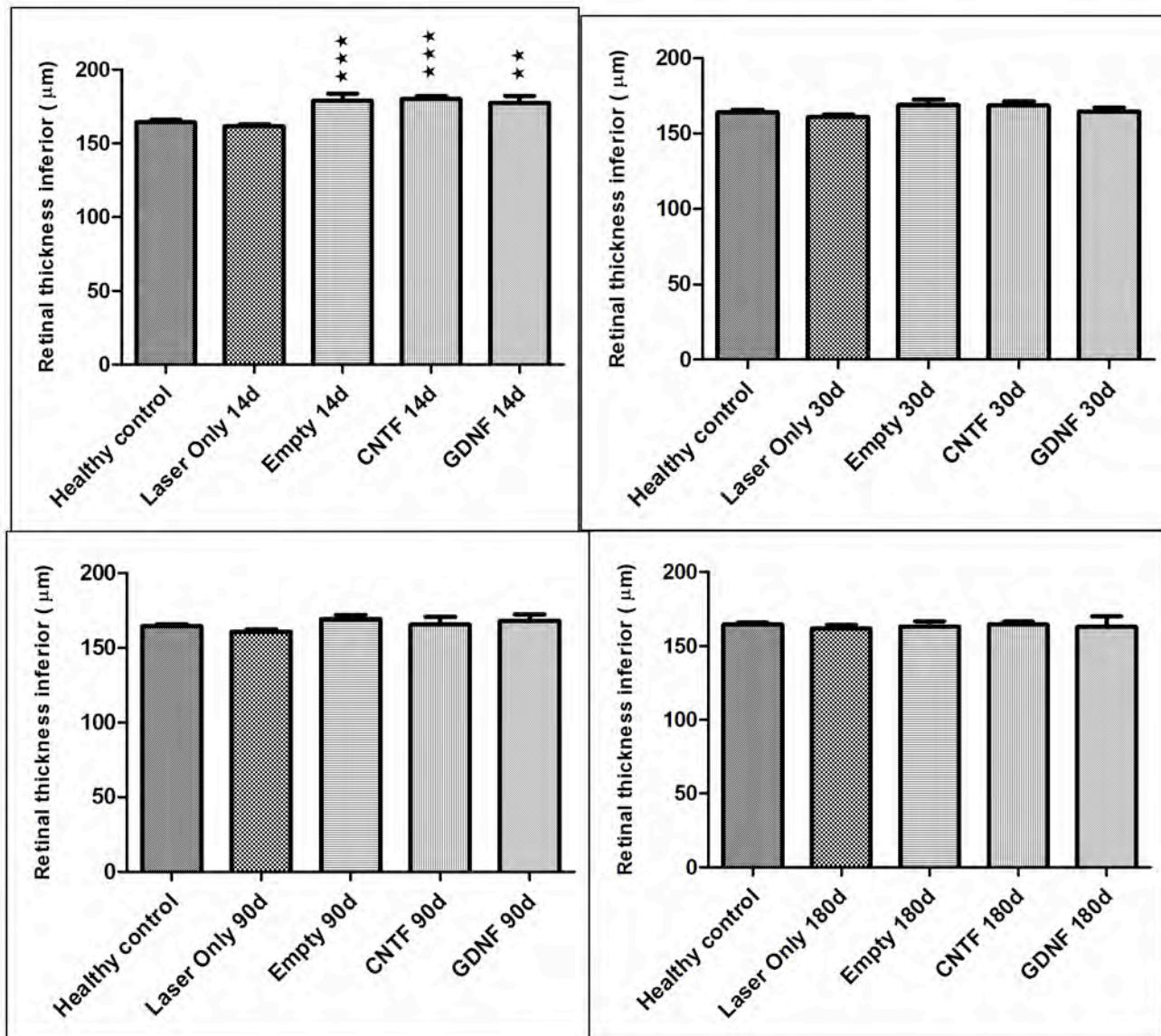
- peripapillary scan (circular scan around optic nerve head, used to assess nerve fiber layer thickness),
- set of linear scans through *area centralis* in superior/tapetal fundus (region located dorso-temporally from the optic nerve head – area of greatest photoreceptor density, corresponds to human macula),
- set of linear scans through inferior/non-tapetal fundus (these scans were aligned vertically with the scans of the superior retina).

Linear scans were used to assess whole retinal thickness, nerve fiber layer thickness and photoreceptor layer thickness. However, only data from the inferior (no-tapetal/pigmented, non-treated) half of the retina of laser-treated eyes was used for statistical analysis (due to multifocal nature of laser damage, it would not be possible to standardize the location of OCT scan lines in the superior retina which was treated with dense array of laser spots).

#### **Total retinal thickness analysis in the inferior (pigmented) retina**

At the first recording time point (14 days after laser exposure), the total retinal thickness in inferior (non-treated) half of retina was increased in all microsphere – injected groups (CNTF, GDNF and empty microspheres), but remained unchanged in laser-only group (Figure 6 – top left graph; unpaired t-test;  $p1<0.0001$  (CNTF),  $p2=0.0022$  (GDNF) and  $p3=0.0006$  (empty microspheres)).

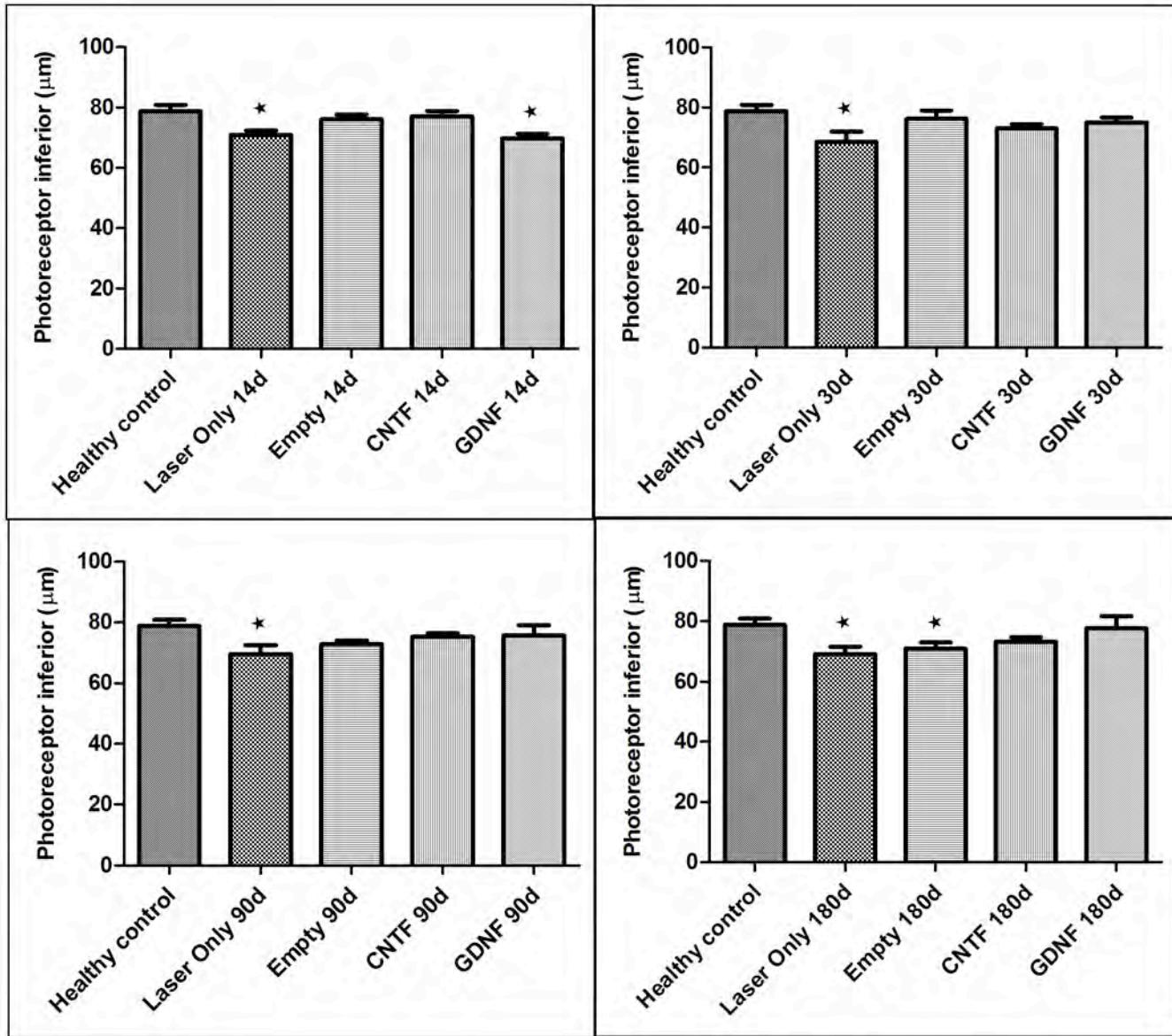
By observation of data from all other recording time points, the total retinal thickness was not changed in any of four groups when compared to values from healthy control animals ( $p>0.05$ ). It is likely that the increase in retinal thickness shortly after laser exposure/intravitreal injection was caused by retinal inflammation/edema caused by cumulative effect of the laser injury and the insult from intravitreal injection and/or rapid microsphere degradation/large growth factor release in the first days after injection rather than by the laser damage itself. CNTF-treated eyes and empty microsphere – treated eyes expressed slightly more severe retinal edema than GDNF-treated eyes. This may suggest that CNTF may be more pro-inflammatory compared to GDNF.



**Figure 6. Analysis of total retinal thickness in inferior (non-treated) half of the retina.** Laser damage was induced in the superior retina of the same eye, while OCT scans were collected through non-tapetal/inferior retina. Significant increase in retinal thickness when compared to control healthy retina was detected in all but laser-only group at the first recording time point (top left graph). However, by 90 days post laser exposure, retinal thickness was unchanged in all four groups and remained so throughout the experiment.

#### **Photoreceptor structural analysis**

Even though not directly affected by the laser energy in the inferior retina (since laser damage was localized to the superior retina), photoreceptor layer thickness in inferior retina of laser-only and empty microsphere injected group decreased significantly over time when compared to values obtained in healthy control dogs (unpaired t-test, p values at 180 days: p1=0.0136 and p2=0.0267 for laser only group and empty microsphere injected group, respectively; Figure 7 – bottom right graph). Dogs that were injected with CNTF/GDNF microspheres had preserved photoreceptor structure throughout the experiment (Figure 7 – bottom right graph).

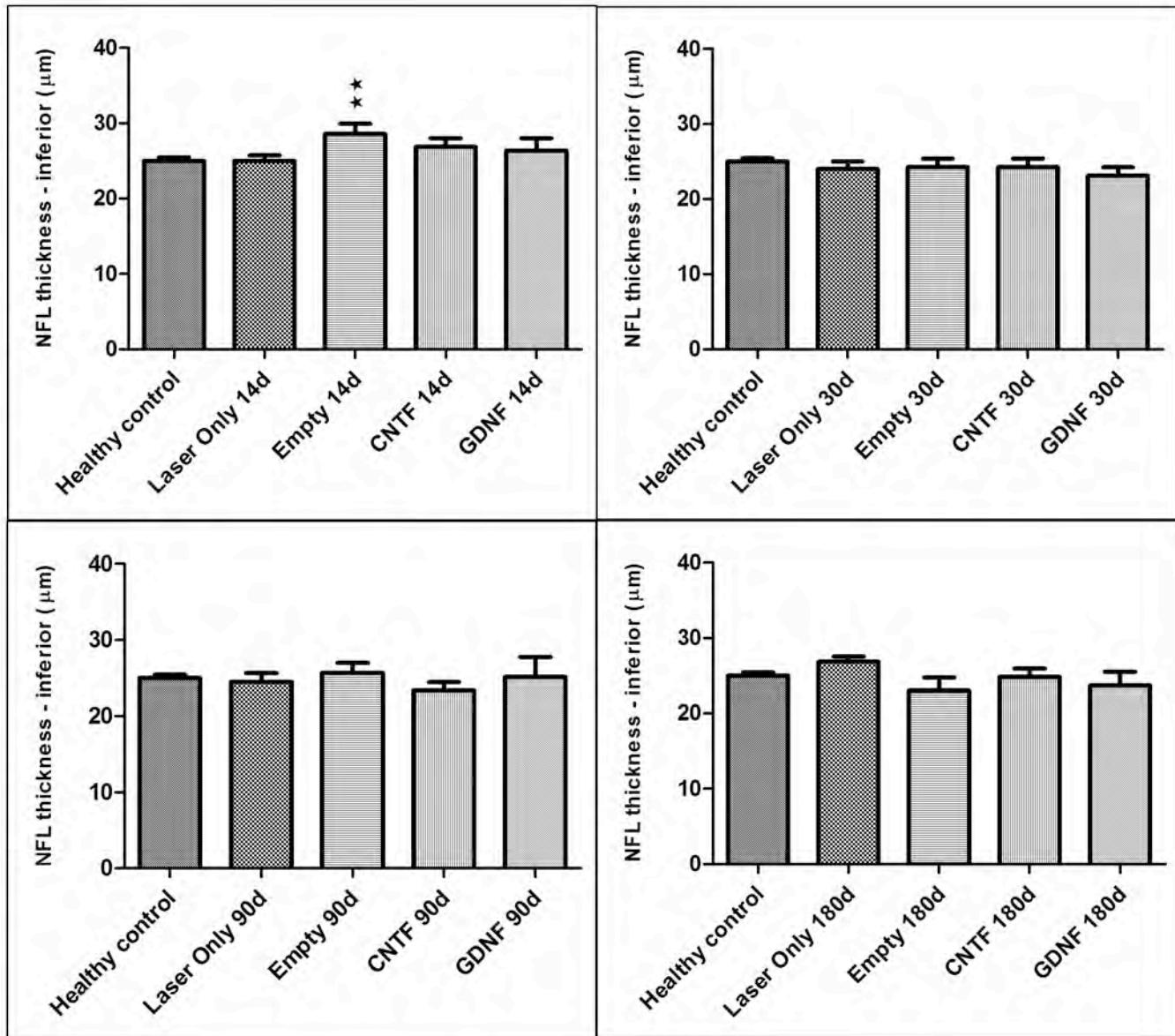


**Figure 7. Photoreceptor layer thickness in inferior (non-laser treated) half of the retina.** Significant decrease in photoreceptor thickness was observed in laser treated eyes when compared to healthy control retinas. GDNF and CNTF treated eyes did not have decreased thickness of the photoreceptor layer at the end of experiment.

#### Nerve fiber layer structural analysis

At the first recording time point (14 days after laser exposure, Figure 8 – top left graph), the nerve fiber layer thickness in inferior retina was significantly increased only in empty microsphere-injected group ( $p=0.0052$ ).

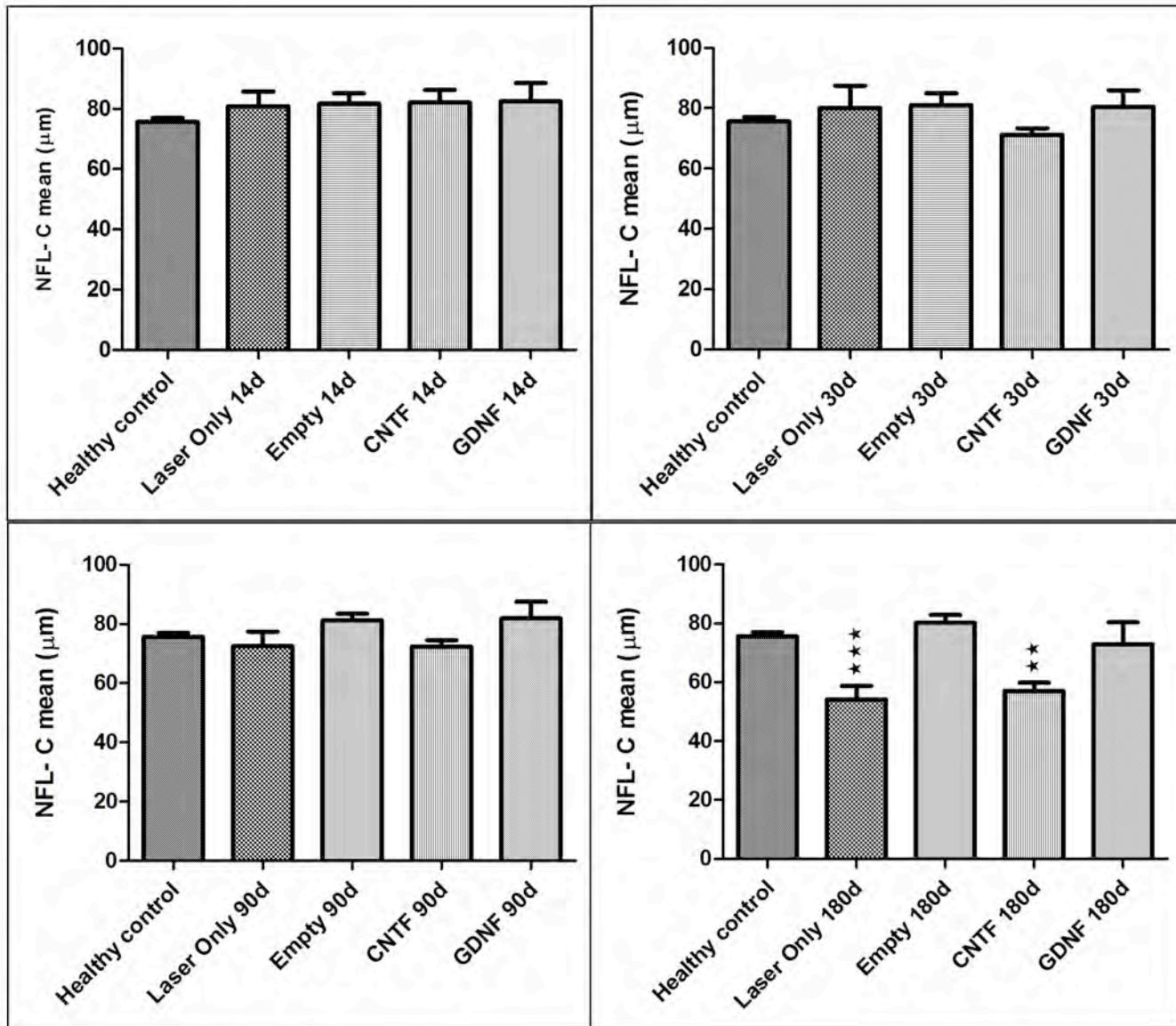
In all later time points, the nerve fiber layer thickness in inferior retina was not changed in any of the four groups when compared to healthy control eyes ( $p>0.05$ ).



**Figure 8.** Nerve fiber layer thickness in inferior (non-treated) half of the retina. Significant increase in nerve fiber layer thickness when compared to healthy control retina was present in empty microsphere – treated group shortly after laser exposure (14 days, top left graph). This was most likely caused by inflammatory edema due to cumulative effect of laser injury and intravitreal injection/rapid microsphere degradation. The nerve fiber layer thickness returned to normal values by 30 days post laser exposure (top right graph) and remained unchanged until the end of experiment in all groups.

#### Peripapillary nerve fiber layer analysis

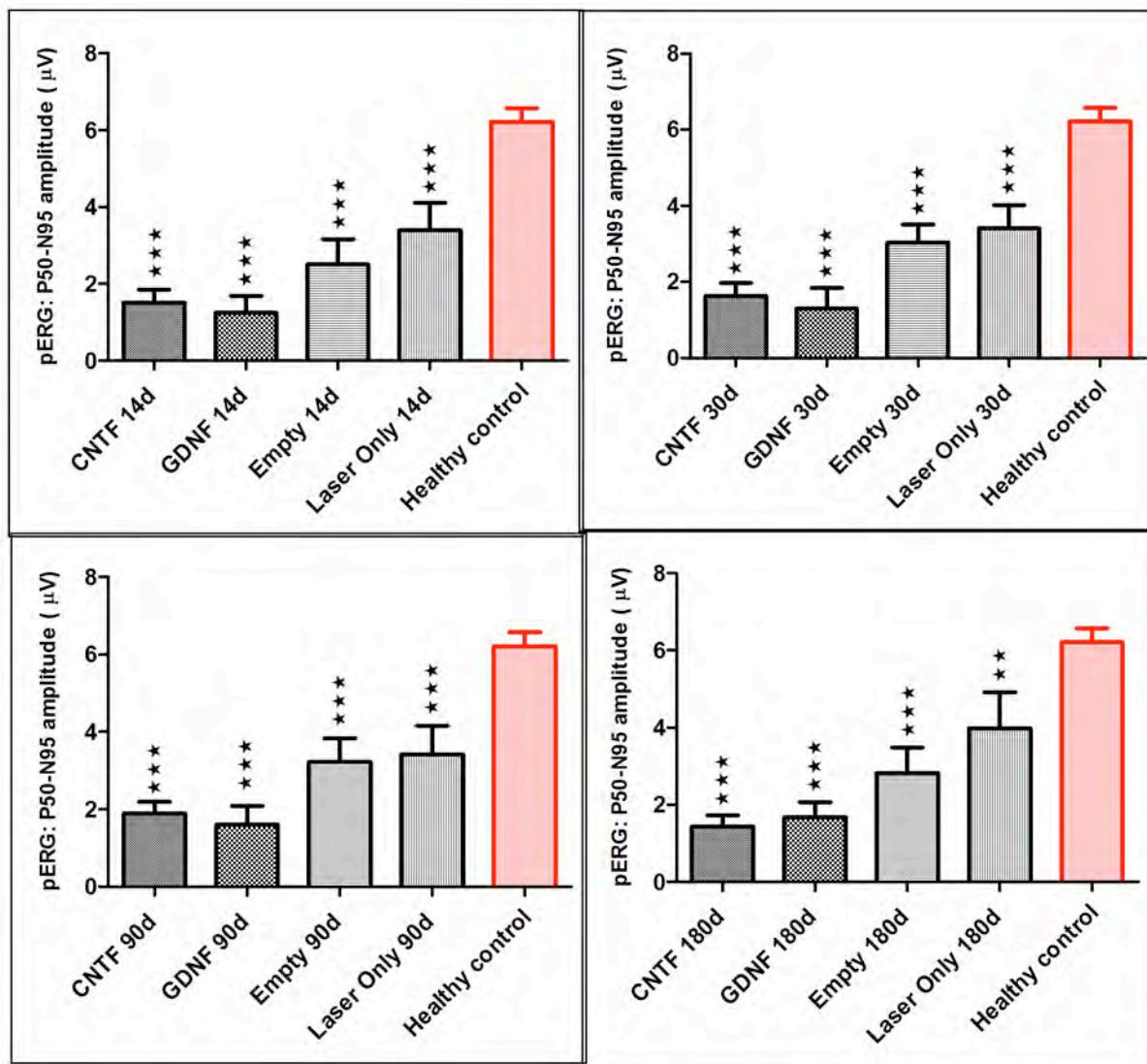
The nerve fiber layer in peripapillary area (mean thickness) was significantly decreased in laser only group and CNTF-treated group 6 months after laser exposure when compared to healthy control dogs (p values for mean peripapillary NFL thickness were <0.0001 in both groups; Figure 9, bottom right graph). GDNF had protective effect on mean peripapillary NFL thickness (180 days after laserinjury, p value was 0.3253, unpaired t-test).



**Figure 9. Peripapillary nerve fiber layer thickness (mean).** Significant decrease in peripapillary nerve fiber layer thickness was present in laser only and CNTF-treated eyes 180 days after laser exposure (bottom right graph). Nerve fiber layer thickness was not changed in GDNF-treated group and empty sphere – injected eyes throughout the experiment.

## 1. Functional characterization of the laser induced damage

**1. Pattern ERG analysis** – pattern ERG (pERG) is an electrophysiological technique, which provides direct and objective evaluation of the retinal ganglion cell (RGC) function. Pattern ERG analysis revealed significant deficits in all laser treated groups at all recording time points (Figure 10). Despite protective effect of GDNF on nerve fiber layer thickness, both CNTF and GDNF seemed to have additive negative effect to laser damage on RGC function (Figure 10; p values at 180 days after laser injury: p1<0.0001 (CNTF), p2<0.0001 (GDNF), p3<0.0001 (empty microsphere) and p4 = 0.0034 (laser only group)).



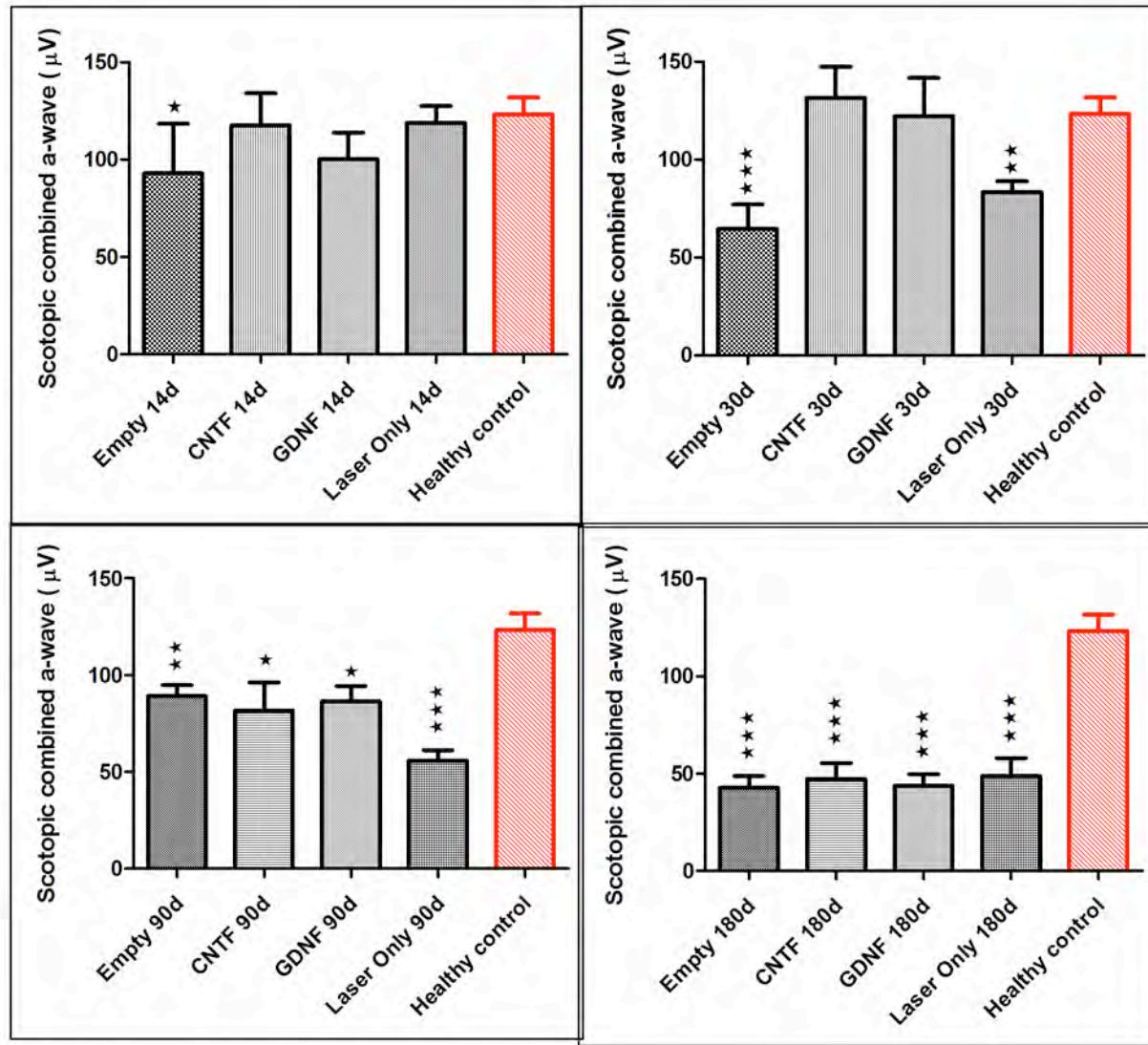
**Figure 10. Pattern ERG analysis of laser treated eyes.** GDNF and CNTF had additional negative effect to laser damage when RGC function was observed.

## 2. Full-field ERG analysis

Full-field ERG analysis was used to evaluate functional status of rods, cones, amacrine cells, bipolar cells and Muller glial cells after laser-induced retinal injury and in combination with different forms of the treatment. ERG analysis showed significant decrease in amplitudes of scotopic a-wave (combined rod-cone function, Figure 11), scotopic b-wave (bipolar cell + Muller cell function, Figure 12) and oscillatory potentials (amacrine cell function, Figure 13) as a result of the laser damage to the retina. Continuous decline of retinal function was noticed even 6 months after initial injury, which may be suggestive of the presence of inflammatory changes and/or progressive retinal degenerative changes even months after initial laser insult.

### Scotopic a-wave

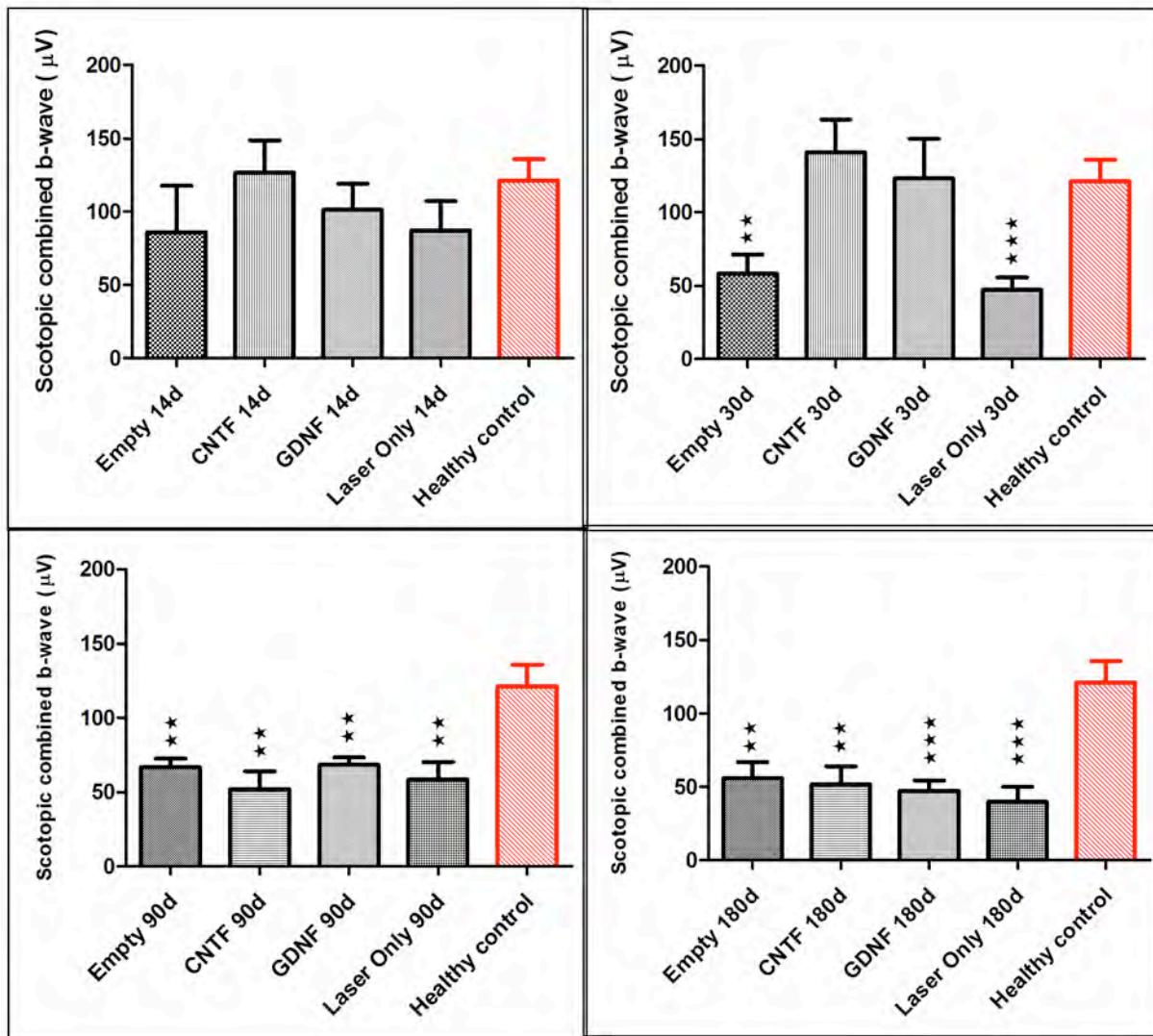
Both GDNF and CNTF treatment showed protective effect in the first 30 days after injury (Figure 11, top right graph; p values were 0.0008 (empty spheres), 0.7824 (CNTF), 0.6662 (GDNF) and 0.0044 (laser only) when compared to healthy untreated eyes). However, the protective effect started to wane off at 90 days after laser injury (Figure 11, bottom left graph; p values were 0.0095, 0.0116, 0.0100 and <0.0001 for empty spheres, CNTF, GDNF and laser only group, respectively) and was completely lost by 180 days after laser injury (Figure 11, bottom right graph; p value for all groups was < 0.0001). This may be result of the absent GDNF and CNTF trophic support due to the complete microsphere degradation by 90 days post injury.



**Figure 11.** Scotopic a-wave – maximum response (combined rod-cone function). Analysis of a-wave amplitude showed significant decrease throughout the experiment in laser only and empty microsphere-treated groups. Both GDNF and CNTF had protective effect on scotopic a-wave amplitude at 30 days after laser exposure (top right graph). However, by 90 days after laser damage, the scotopic a-wave amplitude started to decline even in GDNF and CNTF groups (bottom left graph) and by 180 days after laser exposure, the a-wave deficit was equalized between all groups (bottom right graph).

### Scotopic b-wave

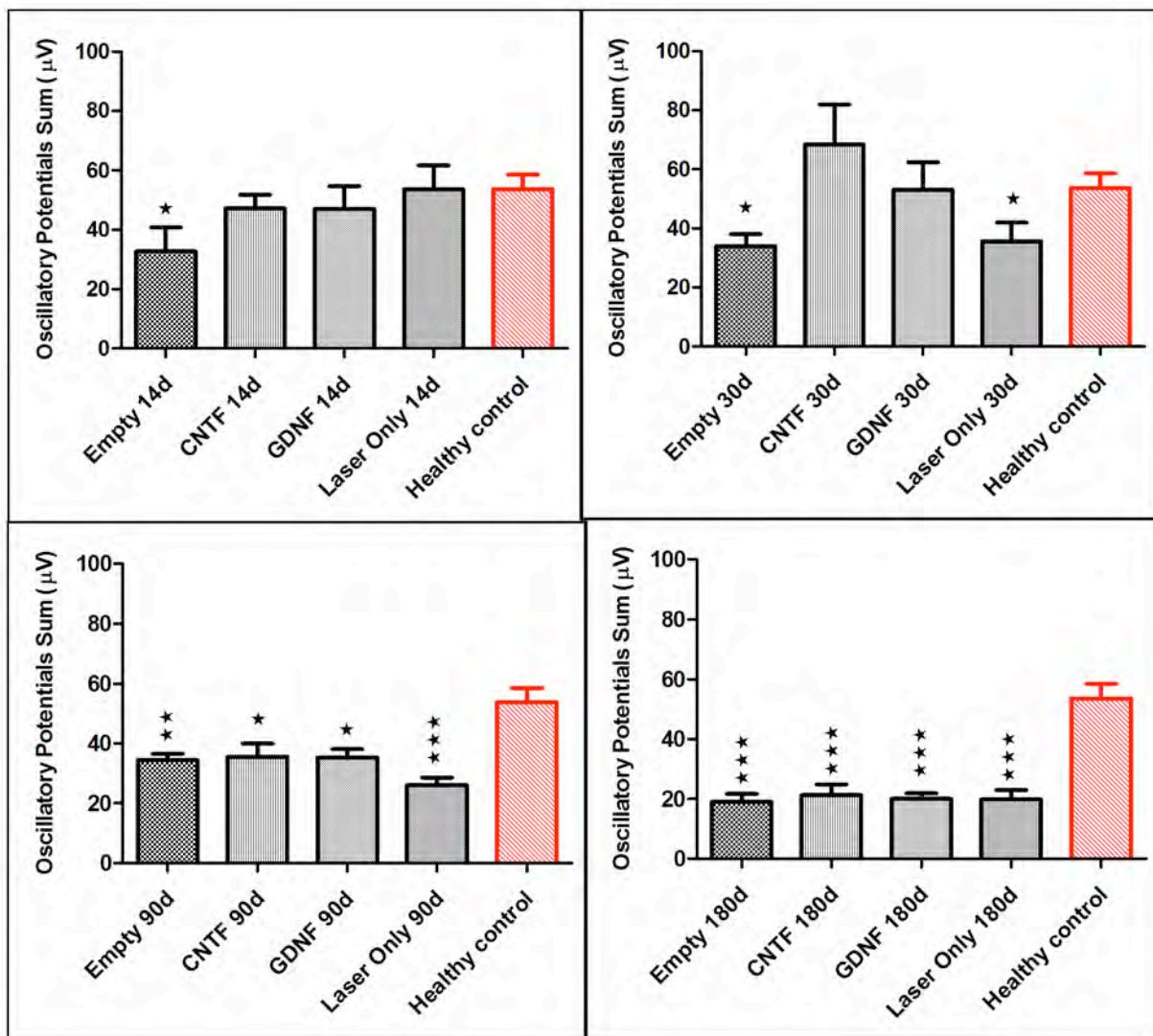
Both GDNF and CNTF treatment showed protective effect in the first 30 days after injury (Figure 12, top right graph; p values were 0.0034, 0.5867, 0.7311 and 0.0004 for empty spheres, CNTF, GDNF and laser only group, respectively). However the protective effect started to wane off at 90 days after laser injury (Figure 12, bottom left graph; p values were 0.0072, 0.0014, 0.0094 and 0.0053 for empty spheres, CNTF, GDNF and laser only group, respectively; unpaired t-test) and was completely lost by 180 days after laser injury (Figure 12, bottom right graph; p values were 0.0053, 0.0025, 0.0002 and 0.0002 for empty spheres, CNTF, GDNF and laser only group, respectively; unpaired t-test). This may be result of the absent GDNF and CNTF trophic support due to the complete microsphere degradation by 90 days post injury.



**Figure 12.** Scotopic b-wave (bipolar cell + Muller cell function). Analysis of b-wave amplitude showed significant decrease throughout the experiment in laser only and empty microsphere-treated groups. Both GDNF and CNTF had protective effect on scotopic b-wave amplitude at 30 days after laser exposure (top right graph). However, by 90 days after laser damage, the scotopic b-wave amplitude declined even in these two groups (bottom left graph) and by 180 days after laser exposure, the b-wave deficit was equalized between the groups (bottom right graph).

### Oscillatory potentials

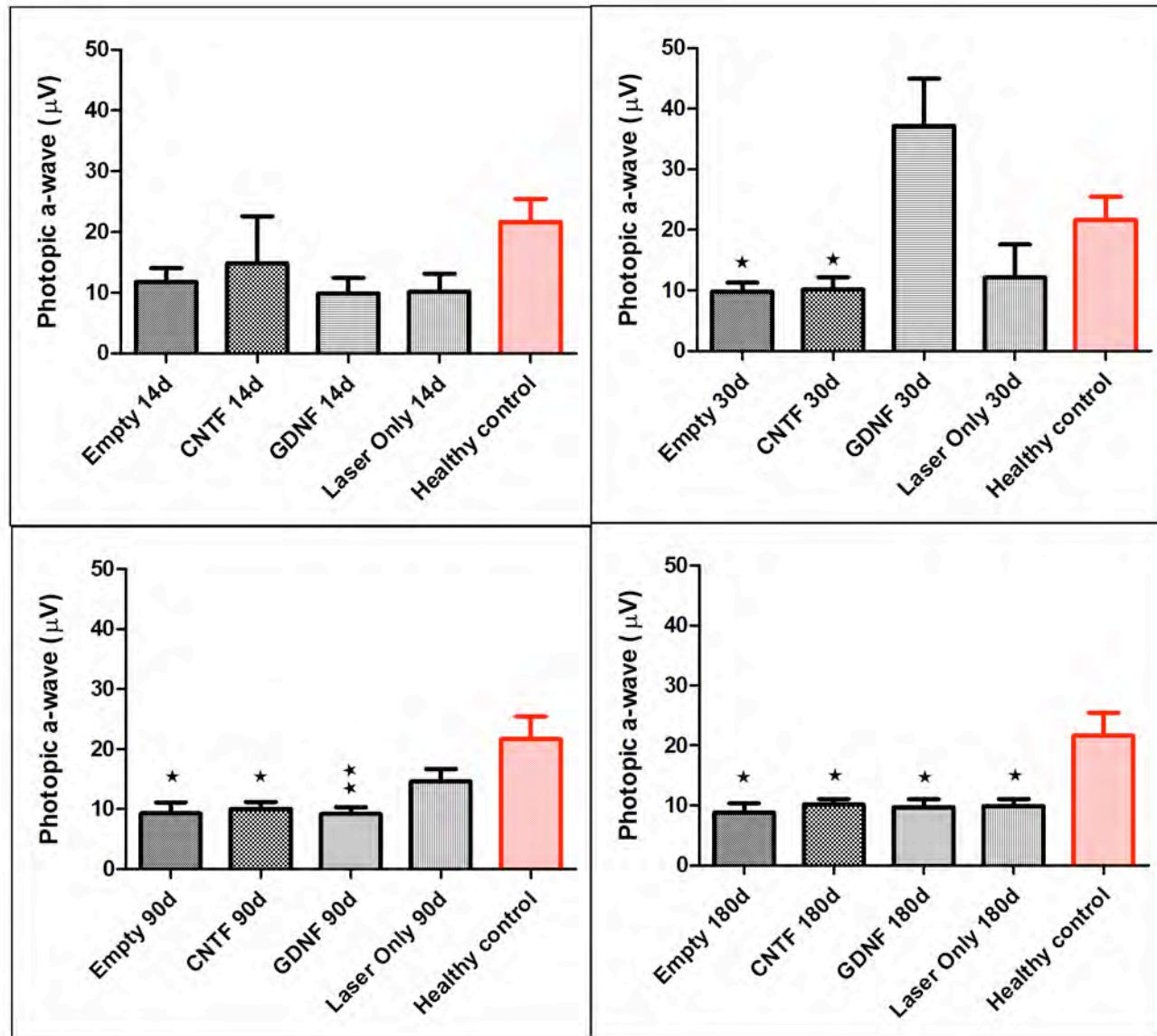
Both GDNF and CNTF treatment showed protective effect in the first 30 days after injury (Figure 13, top right graph; p values were 0.0121, 0.5032, 0.7162 and 0.0253 for empty spheres, CNTF, GDNF and laser only group, respectively; unpaired t-test). However the protective effect started to wane off at 90 days after laser injury (Figure 13, bottom left graph; p values were 0.0078, 0.0166, 0.0118 and 0.0003 for empty spheres, CNTF, GDNF and laser only group, respectively; unpaired t-test) and was completely lost by 180 days after laser injury (Figure 13, bottom right graph; p value for all groups was < 0.0001; unpaired t-test). This may be result of the absent GDNF and CNTF trophic support due to the complete microsphere degradation by 90 days post injury.



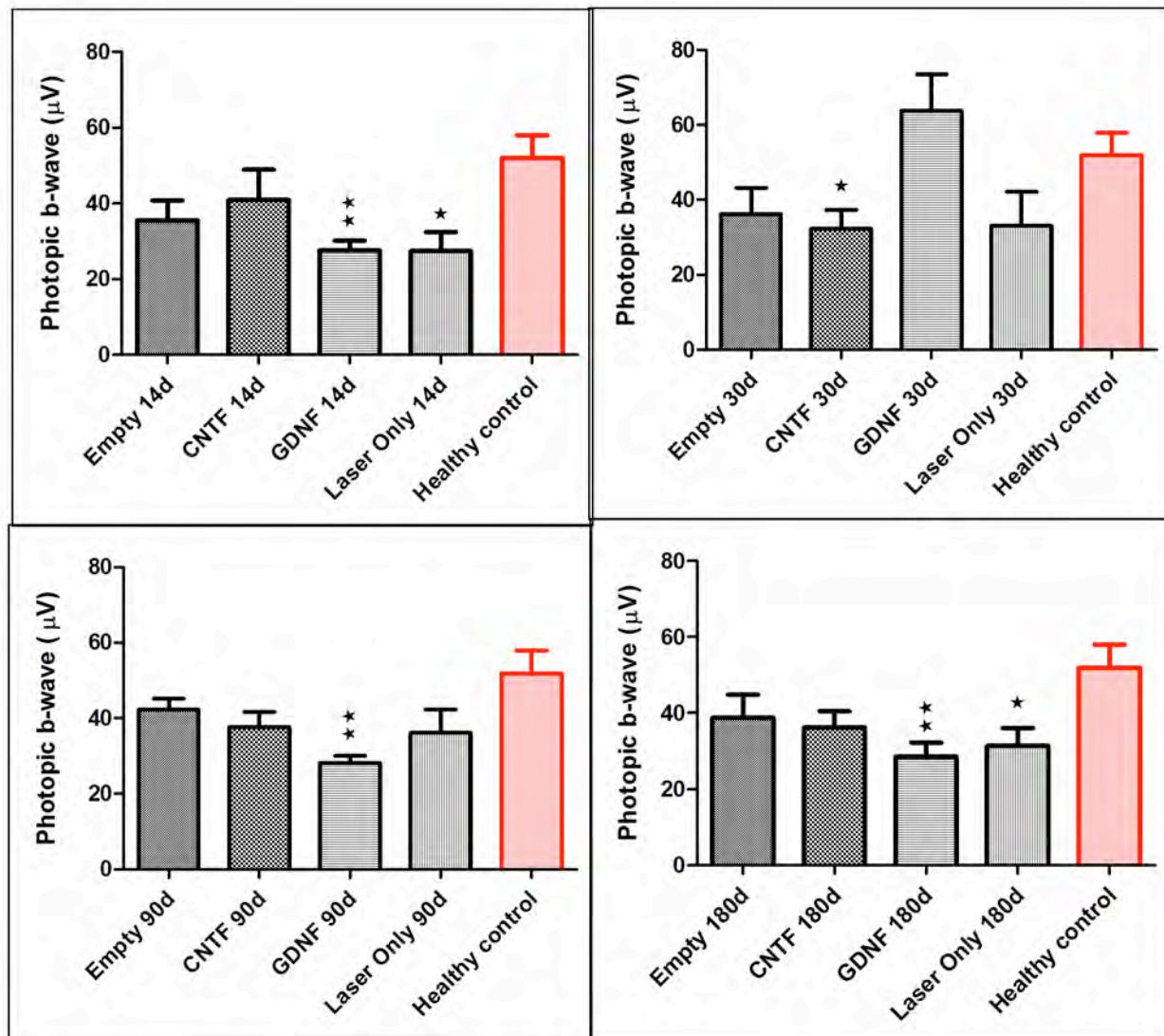
**Figure 13.** Oscillatory potentials (amacrine cell function). Analysis of oscillatory potentials showed significant decrease throughout the experiment in laser only and empty microsphere-treated groups. Both GDNF and CNTF had protective effect on oscillatory potentials at 30 days after laser exposure (top right graph). However, by 90 days after laser damage, the oscillatory potentials declined even in these two groups (bottom left graph) and by 180 days after laser exposure, the oscillatory potentials deficit was equalized between the groups (bottom right graph).

## Evaluation of the cone function in laser injured retinas

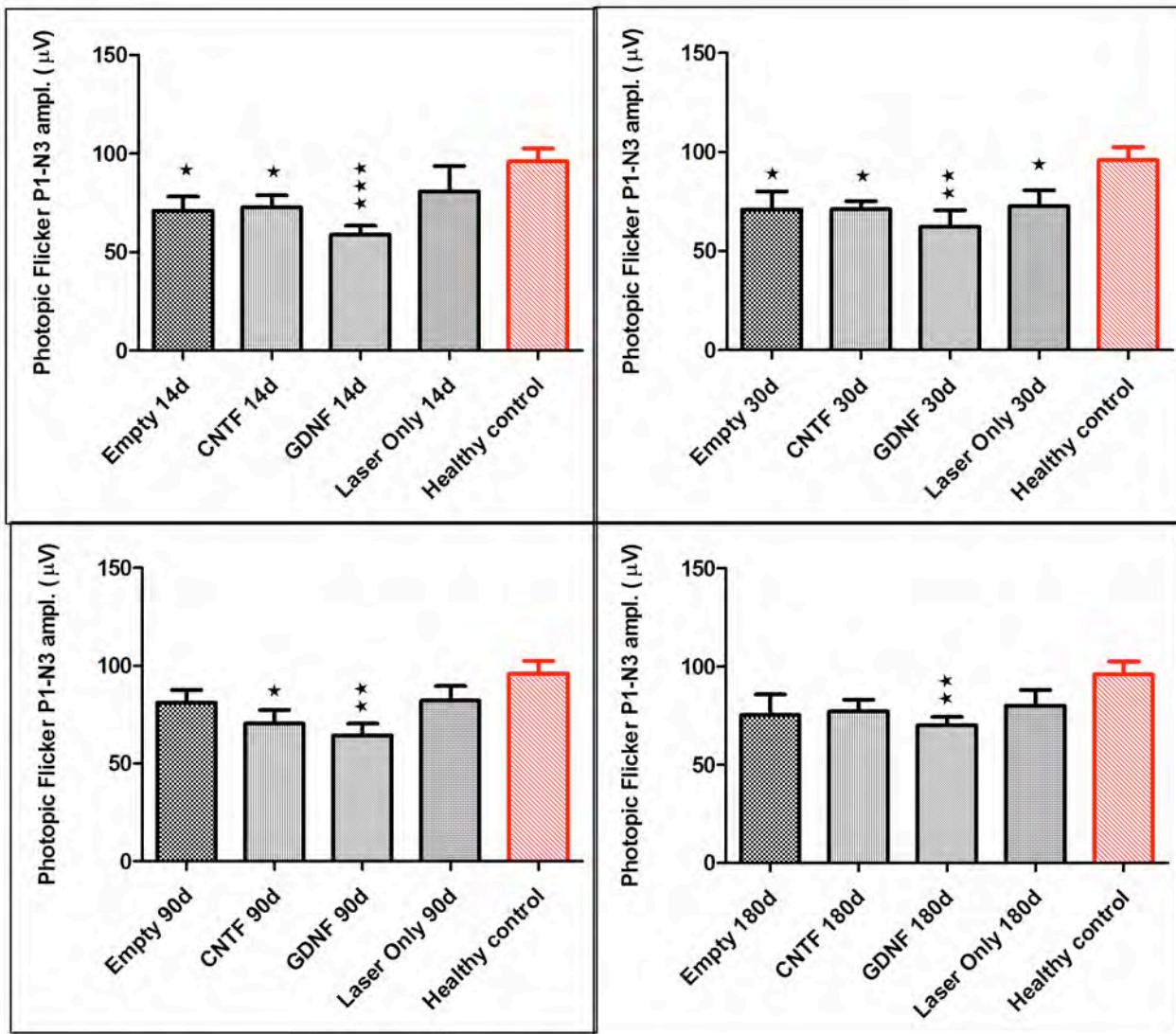
Detailed analysis of cone electrical function revealed decreased function of cones and bipolar cells mediating cone responses in dogs with laser damage to superior retina (Figures 14, 15 and 16).



**Figure 14.** Photopic a-wave: groups at different time points after laser damage. Decrease in a-wave amplitude was induced by laser damage. Despite short-term increase in a-wave amplitude in GDNF-treated group at 30 days (top right graph; p values were 0.0144, 0.0450, 0.1628 and 0.0708 for empty, CNTF, GDNF and laser only group, respectively), a-wave amplitude in this group declined at 90 days (bottom left graph; p values: 0.0147, 0.0157, 0.0081 and 0.2618 for empty, CNTF, GDNF and laser only group, respectively). At 180 days after laser damage, the a-wave deficit was equalized between the groups (bottom right graph; p values: 0.0157, 0.0237, 0.0140 and 0.0211 for empty, CNTF, GDNF and laser only group, respectively).



**Figure 15.** Photopic b-wave: groups at different time points after laser damage. Decrease in b-wave amplitude was induced by laser damage. Despite short-term increase in b-wave amplitude in GDNF-treated group at 30 days (top right graph; p values were 0.1306, 0.0193, 0.4152 and 0.0652 for empty, CNTF, GDNF and laser only group, respectively), in the later time points GDNF seemed to have additive negative effect to laser damage on photopic b-wave. Recordings at 90 days after laser damage is represented in the bottom left graph (p values: 0.2397, 0.0718, 0.0010 and 0.0807 for empty, CNTF, GDNF and laser only group, respectively), recordings at 180 days after laser damage in the bottom right graph (p values: 0.5982, 0.0613, 0.0037 and 0.0226 for empty, CNTF, GDNF and laser only group, respectively).



**Figure 16.** Photopic flicker amplitudes: groups at different time points after laser damage. Decrease in photopic flicker amplitudes was present in all injected eyes at first recording time point (top left graph; p values were 0.0229, 0.0242, 0.0007 and 0.1620 for empty, CNTF, GDNF and laser only group, respectively). At 30 days after laser damage, all groups exhibited decline of photopic flicker amplitude (top right graph; p values were 0.0208, 0.0106, 0.0040 and 0.0332 for empty, CNTF, GDNF and laser only group, respectively). By 180 days after laser damage, the photopic flicker amplitudes normalized in all groups but GDNF. The flicker amplitude in GDNF group remained significantly decreased six months after laser injury (bottom right graph; p values: 0.0959, 0.0535, 0.0070 and 0.1427 for empty, CNTF, GDNF and laser only group, respectively).

## **Supplementary Analysis: Evaluating the relationship between structural and functional properties of the retina and expression of the neurotrophic growth factors GDNF and BDNF after laser injury.**

### **Rationale:**

During the course of this study we have performed a detailed analysis of neurotrophic growth factor expression and associated receptors (BDNF-TrkB, GDNF-GDNFr1 and r2, CNTF-CNTFr1) and performed detailed analysis of retinal functional (electroretinography) and structural parameters (optical coherence tomography) in laser-injured retinas. We have demonstrated that the laser damage to the retina results in an initial decrease of retinal function, which remains relatively static until 14 days post injury, and then a further reduction in retinal function occurs. Furthermore, we have demonstrated that two different neurotrophic growth factors (BDNF and GDNF) are significantly upregulated in laser injured retinas at 3 and 7 days, but not at 14 days post injury which could indicate an intrinsic retinal capacity to preserve its function and structure after a laser insult during the acute period of injury (3-7 days). The further analysis of this data seeks to evaluate the relationship between BDNF and GDNF expression, and how this correlates with the functional and structural properties of the retina after laser injury.

### **Brief Review of the ancillary analysis performed during the no cost extension of this grant:**

Canines were induced with laser injury in the superior or inferior retina and eyes were collected at 3, 7 or 14 days after injury. Laser injury in the inferior region of the retina (more pigmented area without tapetum) induces injury primarily to the outer retina at the RPE-choriod interface, while laser exposure of the superior region of the retina results dominantly in the inner retinal layer injury (due to the lack of RPE pigmentation 810 nm laser energy is primarily absorbed by inner retinal layers in the superior retina)

We have examined the effect of time after laser injury (3, 7 or 14 days) with expression of the neurotrophic growth factors BDNF and GDNF (in the central or peripheral retina), as well as location of the injury (superior vs. inferior retina).

We have correlated expression of BDNF and GDNF with structural retinal measurements of the peripapillary retinal nerve fiber layer: mean overall thickness and regional thickness in the temporal, superior, inferior and nasal quadrants. We have also examined the thickness of the area centralis, which corresponds to the macula of higher vertebrates - these measurements included the mean thickness, the retinal nerve fiber layer thickness (RNFL) and the outer nuclear layer (ONL) thickness.

We also correlated growth factor expression with functional properties of the retina. These included the scotopic Ganzfeld response (Flicker 20 Hz, Flicker P1-N3 amplitude, Cone a-wave, Cone b-wave, and Oscillatory potentials (OSP). We have also examined the pattern ERG response (N35-P50 and P50-N95 components) using temporal stimulation of 2 Hz and 7 Hz to provide information about RGCs.

### **Results:**

We have presented our results in the following tables, with significant ( $p<0.05$ ) positive and negative correlations highlighted as demonstrated below. Following each table is a concise summary of the respective table. At the end of the data, a summary is presented.

Significant positive correlation  $p<0.05$

Significant negative correlation  $p<0.05$

## Correlation of retinal parameters and GDNF expression at 3, 7 and 14 days after laser injury.

### 1. Correlation of retinal GDNF expression with structural parameters of the retina **3 days** post laser injury.

#### 1.A: GDNF expression in the central retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.2114	0.3566	-0.0854	0.1753	0.2038	0.1869	-0.04248	0.4785
P value (one-tailed)	0.2788	0.1559	0.4072	0.314	0.2862	0.3026	0.4536	0.0809
R squared	0.04469	0.1272	0.00730	0.03074	0.04152	0.03492	0.001804	0.2289

#### 1.B: GDNF expression in the peripheral retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.5035	0.5792	0.2875	0.2657	0.5686	0.04187	0.02495	0.2377
P value (one-tailed)	0.069	0.0397	0.2103	0.2291	0.0432	0.4543	0.4727	0.2542
R squared	0.2535	0.3354	0.08265	0.07059	0.3233	0.001753	0.0006223	0.0565

### 2. Correlation of retinal GDNF expression with functional parameters of the retina **3 days** post laser injury.

#### 2.A: GDNF expression in the central retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.3779	-0.2671	-0.6036	0.3037	0.2067	0.6256	0.5235	0.03046	-0.2178
P value (one-tailed)									
R squared	0.1408	0.2279	0.0323	0.1968	0.2833	0.0265	0.0602	0.4667	0.2728

#### 2.B: GDNF expression in the peripheral retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2hz N35-P50	2hz P50-N95	7hz N35-P50	7hz P50-N95
Pearson r	-0.1796	-0.01583	-0.8721	0.3445	0.3153	0.416	0.3634	0.1912	-0.0808
P value (one-tailed)	0.3098	0.4827	0.0005	0.1648	0.1874	0.1159	0.151	0.2983	0.4122
R squared	0.03226	0.0002506	0.7605	0.1187	0.09943	0.173	0.1321	0.03657	0.006

### 3. Correlation of retinal GDNF expression with structural parameters of the retina **7 days** post laser injury.

#### 3.A: GDNF expression in the central retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.047	0.0148	0.1784	-0.0140	-0.0408	-0.3469	-0.2125	-0.2813
P value (one-tailed)	0.4487	0.4838	0.3109	0.4846	0.4554	0.163	0.2778	0.2155
R squared	0.002	0.0002	0.0318	0.0001	0.0016	0.1204	0.0451	0.0791

#### 3.B: GDNF expression in the peripheral retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.3112	0.1855	0.1558	0.1115	0.32	-0.3227	-0.03354	-0.4571
P value (one-tailed)	0.1907	0.304	0.3337	0.3795	0.1837	0.1816	0.4634	0.092
R squared	0.09684	0.0344	0.02428	0.01244	0.1024	0.1041	0.001125	0.209

### 4. Correlation of retinal GDNF expression with functional parameters of the retina **7 days** post laser injury.

#### 4.A: GDNF expression in the central retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	0.4436	0.1886	-0.1796	0.3803	0.5589	0.3806	0.2873	0.1448	0.5057
P value (one-tailed)	0.0995	0.3009	0.3098	0.1392	0.0465	0.139	0.2104	0.345	0.068
R squared	0.1968	0.03556	0.03225	0.1446	0.3124	0.1448	0.08256	0.02095	0.2557

#### 4.B: GDNF expression in the peripheral retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	0.4031	0.05758	0.1157	0.6909	0.2536	0.1822	0.2622	0.2397	0.4499
P value (one-tailed)	0.124	0.4372	0.3752	0.0135	0.2398	0.3072	0.2321	0.2524	0.096
R squared	0.1625	0.003315	0.01338	0.4773	0.06431	0.0332	0.06877	0.05744	0.2024

## 5. Correlation of retinal GDNF expression with structural parameters of the retina **14 days** post laser injury.

### 5.A: GDNF expression in the central retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	-0.1339	0.2075	-0.1672	-0.2542	-0.2045	-0.05949	0.1877	0.04773
P value (one-tailed)	0.7123	0.5652	0.6443	0.4784	0.5708	0.8703	0.6037	0.8958
R squared	0.01792	0.04305	0.02796	0.06464	0.04184	0.003539	0.03521	0.002279

### 5.B: GDNF expression in the peripheral retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.2137	0.5825	0.16	-0.05465	-0.1131	0.116	0.183	-0.1457
P value (one-tailed)	0.2766	0.0386	0.3295	0.4404	0.3779	0.3748	0.3064	0.3439
R squared	0.04568	0.3394	0.02559	0.002986	0.01279	0.01346	0.0335	0.02124

## 6. Correlation of retinal GDNF expression with functional parameters of the retina **14 days** post laser injury.

### 6.A: GDNF expression in the central retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.506	-0.4913	0.1146	-0.5802	-0.1221	-0.4299	-0.512	-0.2617	0.2463
P value (one-tailed)	0.1357	0.1493	0.7525	0.0787	0.7369	0.2149	0.1303	0.4652	0.4927
R squared	0.256	0.2414	0.01314	0.3366	0.0149	0.1848	0.2622	0.06847	0.06067

### 6.B: GDNF expression in the peripheral retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.2394	-0.2087	0.05369	-0.5563	-0.2463	0.3968	0.2546	0.2539	0.4289
P value (one-tailed)	0.2526	0.2814	0.4414	0.0475	0.2463	0.1281	0.2389	0.2395	0.1081
R squared	0.05732	0.04356	0.002883	0.3094	0.06068	0.1575	0.06484	0.06449	0.1839

### Summary of Findings:

- There was a positive correlation between GDNF expression and the peripapillary RNFL thickness at 3 and 14 days post injury.
- GDNF expression had a strong negative correlation with the oscillatory potential response (inner retina) three days after injury.
- A positive correlation was observed for GDNF expression and photoreceptor mediated responses (outer retina; cone function, N35-P50 component of the pERG) at 3 and 7 days after injury.

## Correlation of retinal parameters and BDNF expression at 3, 7 and 14 days after laser injury.

### 7. Correlation of retinal BDNF expression with structural parameters of the retina **3 days** post laser injury.

#### 7.A: BDNF expression in the central retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	-0.5335	-0.4475	-0.3607	-0.5681	-0.4797	-0.7278	-0.5269	-0.8033
P value (one-tailed)	0.0561	0.0973	0.1529	0.0433	0.0803	0.0085	0.0588	0.0026
R squared	0.2847	0.2003	0.1301	0.3227	0.2302	0.5298	0.2777	0.6452

#### 7.B: BDNF expression in the peripheral retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	-0.3774	-0.4677	-0.185	-0.371	-0.3146	-0.6968	-0.5124	-0.8275
P value (one-tailed)	0.1412	0.0864	0.3044	0.1456	0.188	0.0126	0.065	0.0016
R squared	0.1424	0.2187	0.0342	0.1376	0.0989	0.4855	0.2626	0.6847

### 8. Correlation of retinal BDNF expression with functional parameters of the retina **3 days** post laser injury.

#### 8.A: BDNF expression in the central retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	0.7125	0.3738	0.4237	0.1534	0.4421	-0.1062	0.3658	0.4979	0.6401
P value (one-tailed)	0.0104	0.1437	0.1112	0.3361	0.1004	0.3852	0.1493	0.0716	0.0231
R squared	0.5077	0.1397	0.1795	0.02354	0.1955	0.01127	0.1338	0.2479	0.4097

#### 8.B: BDNF expression in the peripheral retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	0.5375	0.08838	0.2724	-0.06806	0.1191	-0.09206	0.2181	0.5521	0.71
P value (one-tailed)	0.0545	0.4041	0.2232	0.4259	0.3715	0.4002	0.2725	0.049	0.0107
R squared	0.289	0.00781	0.0742	0.004632	0.01419	0.008476	0.04756	0.3048	0.5041

## 9. Correlation of retinal BDNF expression with structural parameters of the retina **7 days** post laser injury.

9.A: BDNF expression in the central retina.

	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
Parameter	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.04339	-0.1824	-0.1119	0.07334	0.3076	-0.008083	-0.174	0.1931
P value (one-tailed)	0.4526	0.307	0.3791	0.4202	0.1936	0.4912	0.3153	0.2965
R squared	0.001883	0.03328	0.01252	0.005378	0.09464	0.00006534	0.03028	0.03728

9.B: BDNF expression in the peripheral retina.

	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
Parameter	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.07899	-0.1891	-0.3205	-0.2664	0.6668	0.1972	0.1547	0.1545
P value (one-tailed)	0.4141	0.3004	0.1833	0.2284	0.0176	0.2925	0.3348	0.335
R squared	0.006239	0.03575	0.1027	0.07098	0.4447	0.03891	0.02393	0.02387

## 10. Correlation of retinal BDNF expression with functional parameters of the retina **7 days** post laser injury.

10.A: BDNF expression in the central retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.1517	-0.1733	0.6782	0.2322	-0.3686	-0.09844	0.1607	0.3732	-0.06339
P value (one-tailed)	0.3379	0.3161	0.0156	0.2593	0.1473	0.3934	0.3287	0.1441	0.4309
R squared	0.023	0.03002	0.4599	0.05391	0.1359	0.00969	0.02584	0.1393	0.00401

10.B: BDNF expression in the peripheral retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.2178	0.07775	0.7517	0.6444	-0.5196	-0.3111	-0.003375	0.1695	-0.1925
P value (one-tailed)	0.2728	0.4155	0.0061	0.0221	0.0619	0.1908	0.4963	0.3198	0.297
R squared	0.04743	0.006045	0.565	0.4153	0.2699	0.09679	0.00001139	0.02873	0.03707

## 11. Correlation of retinal BDNF expression with structural parameters of the retina **14 days** post laser injury.

### 11.A: BDNF expression in the central retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.01405	-0.114	0.03522	0.3261	-0.3013	0.1668	-0.08085	0.002811
P value (one-tailed)	0.4846	0.3769	0.4615	0.1789	0.1987	0.3225	0.4121	0.4969
R squared	0.0001975	0.01301	0.001241	0.1063	0.09081	0.02783	0.006537	0.000007902

### 11.B: BDNF expression in the peripheral retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.2559	0.2626	0.2398	0.545	-0.4273	0.9233	-0.4896	0.7066
P value (one-tailed)	0.2377	0.2318	0.2523	0.0516	0.109	P<0.0001	0.0755	0.0112
R squared	0.0655	0.06896	0.05749	0.2971	0.1826	0.8525	0.2397	0.4993

## 12. Correlation of retinal BDNF expression with functional parameters of the retina **14 days** post laser injury.

### 12.A: BDNF expression in the central retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	0.3062	0.3257	0.07732	0.4495	0.3871	0.2154	-0.05408	-0.126	-0.09483
P value (one-tailed)	0.1948	0.1792	0.4159	0.0963	0.1345	0.2751	0.441	0.3644	0.3972
R squared	0.09375	0.1061	0.005978	0.202	0.1499	0.04638	0.002925	0.01587	0.008992

### 12.B: BDNF expression in the peripheral retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	0.6522	0.6551	0.1528	0.3154	0.5848	0.1135	-0.01328	-0.2745	-0.1384
P value (one-tailed)	0.0205	0.0199	0.3367	0.1874	0.0379	0.3774	0.4855	0.2214	0.3514
R squared	0.4253	0.4292	0.02334	0.09946	0.3419	0.01289	0.000176	0.07537	0.01917

### Summary of Findings:

- BDNF expression has a strong negative correlation with structural retina parameters 3 days after injury, which turns to a positive correlation 7 and 14 days after injury.
- Retinal BDNF expression had a positive correlation with functional retina parameters 3, 7 and 14 days post injury. At three days the positive effect was primarily on retinal ganglion cells. At 7 and 14 days post injury the positive effect was on inner and outer nuclear layer cells.

**Correlation of retinal parameters and GDNF expression after inferior retina injury (all time points combined)**

**13. Correlation of retinal GDNF expression with structural parameters of the retina after inferior retina laser injury.**

13.A: GDNF expression in the central retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.08823	0.1152	-0.04299	0.2332	0.01783	-0.2752	-0.4935	0.297
P value (one-tailed)	0.3773	0.3414	0.4395	0.2015	0.4748	0.1605	0.0308	0.1412
R squared	0.007785	0.01326	0.001848	0.05438	0.00031	0.07571	0.2435	0.08819

13.B: GDNF expression in the peripheral retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.2769	0.2818	0.08717	0.3185	0.1411	-0.07437	-0.3111	0.1211
P value (one-tailed)	0.1589	0.1544	0.3787	0.1236	0.308	0.3961	0.1295	0.3337
R squared	0.07668	0.07943	0.007599	0.1015	0.0199	0.005531	0.09678	0.01466

**14. Correlation of retinal GDNF expression with functional parameters of the retina after inferior retina laser injury.**

14.A: GDNF expression in the central retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.1258	-0.1078	-0.003333	0.1545	0.646	0.2086	0.3279	0.2958	-0.0056
P value (one-tailed)	0.3275	0.351	0.4953	0.2912	0.0046	0.2279	0.1164	0.1423	0.4921
R squared	0.01583	0.01163	0.0000111	0.02387	0.4174	0.04349	0.1075	0.08747	0.00003

14.B: GDNF expression in the peripheral retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.3424	0.07106	-0.08683	0.09877	0.3832	0.1818	0.3915	0.4127	0.1201
P value (one-tailed)	0.1058	0.4007	0.3792	0.3631	0.0793	0.2583	0.0745	0.0632	0.3349
R squared	0.1173	0.005049	0.00754	0.009755	0.1468	0.03306	0.1533	0.1703	0.01442

**Summary of Findings:**

- GDNF expression after inferior retina injury had a negative correlation with the RNFL thickness in the area centralis, and a positive correlation with the cone b-wave.

**Correlation of retinal parameters and BDNF expression after inferior retina injury (all time points combined)**

**15. Correlation of retinal BDNF expression with structural parameters of the retina after inferior retina laser injury.**

**15.A: BDNF expression in the central retina.**

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	-0.2868	-0.167	-0.179	-0.08652	-0.413	-0.01999	-0.166	0.1279
P value (one-tailed)	0.15	0.2759	0.2617	0.3796	0.063	0.4718	0.2772	0.3249
R squared	0.08223	0.0279	0.03203	0.007486	0.1706	0.0003996	0.02755	0.01635

**15.B: BDNF expression in the peripheral retina.**

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	-0.3083	-0.3449	-0.145	-0.1389	-0.3302	-0.1954	-0.2012	-0.2436
P value (one-tailed)	0.1318	0.104	0.3031	0.3108	0.1147	0.2427	0.236	0.1908
R squared	0.09502	0.119	0.02101	0.01929	0.1091	0.03817	0.04049	0.05933

**16. Correlation of retinal BDNF expression with functional parameters of the retina after inferior retina laser injury.**

**16.A: BDNF expression in the central retina.**

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.2643	-0.2594	0.7671	-0.03517	-0.1487	-0.32	-0.07039	-0.005906	-0.272
P value (one-tailed)	0.1705	0.1752	0.0004	0.4505	0.2985	0.1225	0.4016	0.4917	0.1633
R squared	0.06987	0.06731	0.5885	0.001237	0.02211	0.1024	0.004955	0.00003489	0.074

**16.B: BDNF expression in the peripheral retina.**

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.2661	-0.3266	0.6119	-0.1021	-0.2536	-0.2184	-0.01714	0.07717	-0.09904
P value (one-tailed)	0.1688	0.1174	0.0077	0.3587	0.1809	0.2171	0.4758	0.3923	0.3627
R squared	0.07083	0.1067	0.3744	0.01042	0.06433	0.04771	0.0002937	0.005955	0.00980

**Summary of Findings:**

- BDNF expression had a strong positive correlation with the oscillatory potential response from the inner retina after inferior retina laser injury.

## Correlation of retinal parameters and GDNF expression after superior retina injury.

### 17. Correlation of retinal GDNF expression with structural parameters of the retina after superior retina laser injury.

#### 17.A: GDNF expression in the central retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.1618	0.2175	0.004194	0.1669	0.05676	0.5416	0.4644	0.5971
P value (one-tailed)	0.2823	0.218	0.4941	0.2761	0.4204	0.0185	0.0406	0.0094
R squared	0.02617	0.04733	0.0000175	9	0.02786	0.003222	0.2933	0.2157

#### 17.B: GDNF expression in the peripheral retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.4336	0.4637	0.2915	0.3077	0.272	0.4931	0.6751	0.4057
P value (one-tailed)	0.0532	0.0408	0.1459	0.1323	0.1634	0.0309	0.0029	0.0668
R squared	0.188	0.215	0.08497	0.0947	0.07399	0.2432	0.4558	0.1646

### 18. Correlation of retinal GDNF expression with functional parameters of the retina after superior retina laser injury.

#### 18.A: GDNF expression in the central retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.4106	-0.4046	-0.1452	0.03333	-0.3113	0.5982	0.31	-0.08826	-0.3905
P value (one-tailed)	0.0642	0.0673	0.3028	0.4531	0.1294	0.0093	0.1304	0.3772	0.075
R squared	0.1686	0.1637	0.02109	0.001111	0.09691	0.3578	0.09613	0.00779	0.1525

#### 18.B: GDNF expression in the peripheral retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.2926	-0.2588	-0.115	0.01797	-0.2586	0.3281	0.1698	0.109	-0.2591
P value (one-tailed)	0.1449	0.1758	0.3417	0.4747	0.176	0.1163	0.2725	0.3495	0.1755
R squared	0.08563	0.06699	0.0132	0.0003228	0.0668	0.1076	0.02885	0.01188	0.06714

#### Summary of Findings:

- GDNF expression after superior retina laser injury was positively correlated with structural parameters in the peripapillary region and in the area centralis of the retina.
- There was a positive correlation with GDNF expression on the N35-P50 component of the pERG response.

## Correlation of retinal parameters and BDNF expression after superior retina injury.

### 19. Correlation of retinal BDNF expression with structural parameters of the retina after superior retina laser injury.

#### 19.A: BDNF expression in the central retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	-0.1709	-0.2389	-0.1836	-0.259	0.1202	-0.3086	-0.3418	-0.203
P value (one-tailed)	0.2713	0.1956	0.2562	0.1757	0.3348	0.1315	0.1062	0.2341
R squared	0.0292	0.05707	0.03371	0.06707	0.01444	0.09525	0.1169	0.0412

#### 19.B: BDNF expression in the peripheral retina.

Parameter	Peripapillary Retinal Nerve Fiber Layer					Area Centralis		
	Mean Thickness	Temporal Quadrant	Superior Quadrant	Nasal Quadrant	Inferior Quadrant	Mean Thickness	RNFL Thickness	ONL
Pearson r	0.1005	-0.01606	-0.06059	-0.07552	0.4885	-0.1761	-0.1866	-0.1641
P value (one-tailed)	0.3608	0.4773	0.4151	0.3945	0.0323	0.2651	0.2527	0.2795
R squared	0.0101	0.000258	0.003671	0.005703	0.2387	0.03101	0.03483	0.02692

### 20. Correlation of retinal BDNF expression with functional parameters of the retina after superior retina laser injury.

#### 20.A: BDNF expression in the central retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	-0.01643	0.01454	0.23	0.3313	-0.08862	-0.005464	-0.002902	0.1668	0.1867
P value (one-tailed)	0.4768	0.4795	0.2048	0.1139	0.3767	0.4923	0.4959	0.2762	0.2527
R squared	0.000269	9	0.0002113	0.0528	9	0.1097	0.0078	0.000029	0.0000084
									0.0348
									4

#### 20.B: BDNF expression in the peripheral retina.

Parameter	Flicker 20 Hz	Flicker P1-N3	OSP	Cone a-wave	Cone b-wave	2 Hz N35-P50	2 Hz P50-N95	7 Hz N35-P50	7 Hz P50-N95
Pearson r	0.06022	0.07401	0.2403	0.585	-0.2911	-0.08608	0.03136	0.4383	0.2876
P value (one-tailed)	0.4156	0.3966	0.1941	0.011	0.1462	0.3802	0.4558	0.0511	0.1493
R squared	0.003626	0.005478	0.0577	0.3423	0.0847	0.00741	0.00098	0.1921	0.0827

#### Summary of Findings:

- A positive correlation between BDNF and the peripapillary RNFL was observed after superior retinal injury.
- A positive correlation between BDNF and the Cone a-wave was observed after superior injury.

### **Conclusions from the ancillary study performed this past year:**

- 1) Laser damage to a region of the retina (in this model of canine damage) results in up-regulation of growth factors BDNF and GDNF diffusely across the retina, even in areas not exposed to laser. This may be a result of a diffuse response of the retina to focal injury.
- 2) There was a time dependent association between growth factor expression in the retina and whether an increase or decrease in function of the outer or inner retina was found. This may be due to the pleiotrophic properties of these growth factors, namely, the increase or decrease in function is concentration dependent. Too much growth factor can reduce function due to the nature of their receptor binding and which of their recipient binding sites are activated. Some of their receptors have a deleterious effect when bound and others have a therapeutic effect. Therefore, time dependent changes in function that are biphasic may be explained by how the concentration of these factors varies after time from laser injury and which of their receptors are activated. This has important implications on future treatment with growth factors or agents that stimulate their receptors – there is a need for receptor specific agents that preferentially activate receptors that improve function.
- 3) Time after injury may be the most determinant factor in deciding on therapeutic interventions to reduce permanent visual loss after laser injury, since the response to injury is diffuse and time dependent. Adequate therapeutic strategies using neurotrophic growth factors should not interfere with intrinsic changes in their expression (or expression of their respective receptors), but should have synergistic effect. This can be effectively achieved by selecting the specific timing and the type of growth factors, which will result in the effective binding to the present receptors and will not compete with already present (intrinsically produced) neurotrophic growth factors.

## **Statement of Work:**

Treatment of Laser-Induced Retinal Injury (LIRI) and Visual Loss Using Sustained Release of Intra-vitreal Neurotrophic Growth Factors

Randy H. Kardon, M.D. Ph.D., Investigator-Initiated Research Award

### **Description of the work to be accomplished for specific aims:**

Specific Aim 1: *The molecular profile of intrinsic growth factors and their receptors in dog eyes with laser-induced retinal damage will be characterized in order to identify the appropriate neurotrophic growth factor candidate(s) for neuroprotective therapy.* This group will be subdivided in 2 groups of 15 dogs. The first subgroup will receive laser burns (100 spots, 100 mW pulse energy and 200 ms pulse duration) in the inferior (non-tapetal) retina with a goal of damaging the photoreceptors and retinal pigment epithelium. The second group will receive the laser treatment (100 spots, 150 mW pulse energy and 200 ms pulse duration) in the superior (tapetal) retina with a goal of damaging the nerve fiber layer. By adopting this strategy, we will be able to investigate laser effects on the outer retina and nerve fiber layer in the same experimental system (canine eye). After laser treatment, eyes will be collected at 3, 7 and 15 days postoperatively (n=5 per time point for each group) for analysis of neurotrophic factors (BDNF, CNTF, GDNF) and their respective receptors in the retina.

Timeline: These experiments will be performed in year 1 (1-01-2007 to 12-31-2007).

Milestones: a) completion of the laser-induced damage in the less pigmented tapetal retina in 15 dogs and the pigmented non-tapetal retina in 15 dogs, b) collection of retina tissue from treated eyes and opposite non-treated eyes at 3, 7, and 15 days after laser treatment (5 animals per time point), c) analysis of growth factor levels and receptor levels in retina tissues collected from each eye and at each time point, d) construct summary table of growth factor level and receptors for BDNF, CNTF, and GDNF.

Specific Aim 2: *The degree of protection and recovery will be determined in a canine experimental model of laser-induced retinal damage by treatment with neurotrophic growth factor(s) injected into the vitreous.* A total of 49 dog eyes having acute laser-induced retinal damage will be treated with intravitreal injection of growth factors and will be divided into two major laser-damaged groups receiving growth factors or placebo intravitreal injections; 1) 21 dogs laser treated in non-tapetal retinal areas having relatively heavy pigmentation, causing absorption of laser light primarily in the outer retina containing photoreceptors and 2) 28 dogs laser treated in tapetal retina having sparse pigmentation, causing more absorption of laser light and damage to the inner retina. This will allow us to determine how the degree of retinal pigmentation affects the degree of laser damage and recovery with treatment, analogous to humans with differing eye pigmentation. Furthermore, we will be able to evaluate the neuroprotective treatment for the inner and outer retina damage which can result in humans exposed to laser weapons with capabilities of rapidly changing wavelength and power.

The pigmented eye treatment group will consist of 21 dogs (receiving laser in the inferior, pigmented, non-tapetal retina to induce the photoreceptor and retinal pigment epithelial (RPE) damage) and 28 dogs in the non-pigmented treatment group (receiving laser to the superior lightly pigmented non tapetal retina). Treatment will consist of an intravitreal injection of growth factors (control;n=7, CNTF;n=7, or BDNF;n=7 in the pigmented eyes; control;n=7, CNTF;n=7, BDNF;n=7, or GDNF;n=7 in the non-pigmented eyes). The number of growth factor treatment groups and the number of animals in each treatment group may be modified, based on the information that we obtain from Specific Aim 1 regarding the pattern of expression of the different growth factors and their receptors in the retina. Intravitreal injection will occur within 6 hours after laser-induced injury. We anticipate that a 6 hour post-injury treatment window is a realistic time frame for the intravitreal application of the therapeutic factors which would occur under battlefield conditions. All treatment subgroups will be assessed by *in vivo* functional

and morphological recordings preoperatively, at 15 days postoperatively and 1, 3 and 6 months postoperatively (the necessity of the 6 month time point will be determined and reassessed based on whether any significant difference is detected between the 1 month and 3 month time points. At the end of experiment all eyes from treated animals will be collected for histological analysis.

**Timeline:** The experiments for the pigmented and non-pigmented group of laser treated dog eyes receiving intravitreal growth factors or placebo will be performed in years 2 and 3 (1-01-2008 to 12-31-2009), including *in vivo* testing of vision, structure, and histology.

**Milestones:** a) laser-induced damage in pigmented and non-pigmented areas of retina, b) intravitreal injection of sustained release growth factors within 6 hours after laser-induced damage, c) *in vivo* measurement of visual function and structure pre and post-treatment (15 days, 1, 3, and 6 months), d) histological correlation with *in vivo* functional and structural assessment.

**Methods:** Adult laboratory Beagle dogs will be anesthetized with isoflurane. Diode laser (Iris Medical, 810 nm wavelength) will be used after pupil dilation to place 100 evenly spaced focal burns in the superior (tapetal) or inferior (non-tapetal) retina with a goal of inducing damage to the nerve fiber layer or outer retina, depending on the treated location. Functional testing of vision before and after laser treatment and at different time points after growth factor injection will consist of pupillography to quantify the relative afferent pupil defect, and recording of evoked potentials (Ganzfeld electroretinography, ERG; multifocal electroretinography, mfERG and pattern electroretinography, PERG). Retinal structural damage will be assessed *in vivo* using retinal photography and optical coherence tomography (OCT) and correlated with histological analysis.

**Outcomes for each phase of the project:** 1) table summarizing pattern of presence of intrinsic growth factors and their receptors at different time points after laser-induced retinal damage in laser treated eyes without exogenous injection of growth factors, 2) Comparison of different neuro-trophic growth factor treatment effects on visual function and structure in laser-damaged eyes compared to placebo treated laser damaged eyes, 3) assessment of how treatment effects of intravitreal injection of exogenous growth factors correlate with pattern of intrinsic growth factors and their receptors.

#### **Study site information:**

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Markus Kuehn, Ph.D. Research Associate, Molecular Ophthalmology Laboratory  
Miriam Bridget Zimmerman Ph.D., Director of Statistical Consulting  
No animal or human use at this site for grant

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Sinisa Grozdanic, DVM, PhD  
Matt Harper, Post doctoral fellow  
Animal use at this site for grant



**REPORT OF INVENTIONS AND SUBCONTRACTS**  
*(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)*

Form Approved  
 OMB No. 9000-0095  
 Expires Aug 31, 2001

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (9000-0095), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

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1.a. NAME OF CONTRACTOR/SUBCONTRACTOR University of iowa	c. CONTRACT NUMBER W81XWH-07-1-0686	2.a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT(X one) <input type="checkbox"/> a. INTERIM <input checked="" type="checkbox"/> b. FINAL
b. ADDRESS (Include ZIP Code)	d. AWARD DATE (YYYYMMDD) 2007/10/23	b. ADDRESS (Include ZIP Code)	d. AWARD DATE (YYYYMMDD)	4. REPORTING PERIOD (YYYYMMDD) a. FROM 2007/10/23 b. TO 2011/10/23

**SECTION I - SUBJECT INVENTIONS**

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR (If "None," so state)

NAME(S) OF INVENTOR(S) (Last, First, Middle Initial)  a.	TITLE OF INVENTION(S)  b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER  c.	ELECTION TO FILE PATENT APPLICATIONS (X) d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER (X) e.	
			(1) UNITED STATES	(2) FOREIGN	(a) YES	(b) NO	(a) YES	(b) NO
Kardon, Randy H	no invention to disclose		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR

(1) (a) NAME OF INVENTOR (Last, First, Middle Initial)	(2) (a) NAME OF INVENTOR (Last, First, Middle Initial)	(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
(c) ADDRESS OF EMPLOYER (Include ZIP Code)	(c) ADDRESS OF EMPLOYER (Include ZIP Code)		

**SECTION II - SUBCONTRACTS (Containing a "Patent Rights" clause)**

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR (If "None," so state)

NAME OF SUBCONTRACTOR(S)  a.	ADDRESS (Include ZIP Code)  b.	SUBCONTRACT NUMBER(S)  c.	FAR "PATENT RIGHTS" d.		DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e.	SUBCONTRACT DATES (YYYYMMDD) f.	
			(1) CLAUSE NUMBER	(2) DATE (YYYYMM)		(1) AWARD	(2) ESTIMATED COMPLETION
Iowa State University						2007/10/23	2010/10/23

**SECTION III - CERTIFICATION**

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR (Not required if: (X as appropriate))  SMALL BUSINESS  NON-PROFIT ORGANIZATION

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL (Last, First, Middle Initial)	b. TITLE	c. SIGNATURE	d. DATE SIGNED
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## DD FORM 882 INSTRUCTIONS

### GENERAL

This form is for use in submitting INTERIM and FINAL invention reports to the Contracting Officer and for use in reporting the award of subcontracts containing a "Patent Rights" clause. If the form does not afford sufficient space, multiple forms may be used or plain sheets of paper with proper identification of information by item number may be attached.

An INTERIM report is due at least every 12 months from the date of contract award and shall include (a) a listing of "Subject Inventions" during the reporting period, (b) a certification of compliance with required invention identification and disclosure procedures together with a certification of reporting of all "Subject Inventions," and (c) any required information not previously reported on subcontracts containing a "Patent Rights" clause.

A FINAL report is due within 6 months if contractor is a small business firm or domestic nonprofit organization and within 3 months for all others after completion of the contract work and shall include (a) a listing of all "Subject Inventions" required by the contract to be reported, and (b) any required information not previously reported on subcontracts awarded during the course of or under the contract and containing a "Patent Rights" clause.

While the form may be used for simultaneously reporting inventions and subcontracts, it may also be used for reporting, promptly after award, subcontracts containing a "Patent Rights" clause.

Dates shall be entered where indicated in certain items on this form and shall be entered in six or eight digit numbers in the order of year and month (YYYYMM) or year, month and day (YYYYMMDD). Example: April 1999 should be entered as 199904 and April 15, 1999 should be entered as 19990415.

- 1.a. Self-explanatory.
- 1.b. Self-explanatory.
- 1.c. If "same" as Item 2.c., so state.
- 1.d. Self-explanatory.
- 2.a. If "same" as Item 1.a., so state.
- 2.b. Self-explanatory.
- 2.c. Procurement Instrument Identification (PII) number of contract (DFARS 204.7003).
- 2.d. through 5.e. Self-explanatory.

5.f. The name and address of the employer of each inventor not employed by the contractor or subcontractor is needed because the Government's rights in a reported invention may not be determined solely by the terms of the "Patent Rights" clause in the contract.

Example 1: If an invention is made by a Government employee assigned to work with a contractor, the Government rights in such an invention will be determined under Executive Order 10096.

Example 2: If an invention is made under a contract by joint inventors and one of the inventors is a Government employee, the Government's rights in such an inventor's interest in the invention will also be determined under Executive Order 10096, except where the contractor is a small business or nonprofit organization, in which case the provisions of 35 U.S.C. 202(e) will apply.

- 5.g.(1) Self-explanatory.
- 5.g.(2) Self-explanatory with the exception that the contractor or subcontractor shall indicate, if known at the time of this report, whether applications will be filed under either the Patent Cooperation Treaty (PCT) or the European Patent Convention (EPC). If such is known, the letters PCT or EPC shall be entered after each listed country.
- 6.a. Self-explanatory.
- 6.b. Self-explanatory.
- 6.c. Self-explanatory.
- 6.d. Patent Rights Clauses are located in FAR 52.227.
- 6.e. Self-explanatory.
- 6.f. Self-explanatory.
- 7. Certification not required by small business firms and domestic nonprofit organizations.
- 7.a. through 7.d. Self-explanatory.